CHARACTERISATION OF THE *cysQ* LOCUS OF *Escherichia coli: cysQ* IS ACTIVATED BY ENTRY INTO STATIONARY PHASE.

MICHAEL DAVID MCDONALD

1993

Thesis submitted in partial fulfilment of the degree of
Master of Science in Cellular and Molecular Biology,
University of Canterbury.

ACKNOWLEDGEMENTS

In acknowledging the many people to whom I have become indebted during the course of this thesis, I would like to comment on the extent to which finishing my thesis has been a lesson in how little one can do by oneself. Without a doubt I could not have finished without all of your help. Firstly I would like to thank my supervisor, Khris Mahanty, for his supervision, especially during the period of my write-up, and for having confidence in my work. I would also like to thank Abanti Mahanty for always being so hospitable. Thanks also to Lorraine Fellowes and Beth Robson, both have whom have done so much to make the lab a pleasant place to be. A huge thank you to my work mates Fiona, Jo, Steve, Mark, Graham and Glenn, all of whom resulted in the day-to-day work environment that kept me sane.

My thanks are also due to Alex McLellan who did the earlier work which provided the framework for this study. I would also like to acknowledge Barbara Bachmann and Michael Fath who provided essential bacterial strains, Andrew Fellowes for the PCR primers, Martin Kennedy for generously giving me ^{35}S-ATP, and Barbara Peddie and Steve Chambers who both helped out in the early stages of my project. Thank you also to Jackie Aislabie and Barry Palmer who both acted as co-supervisors, and to Roberto Kolter and especially Bob Baxter, who both provided extremely significant suggestions which have affected the outcome of this thesis.

I would like to give thanks to my family and friends who have given me support through the last, at times very difficult, two years. Jenny Rockel and David Gunby have supported me, both physically and emotionally, as if I was their own son. My mother, Maureen Frew, has made her support for me clear, despite having seen little of me over the period of this thesis. I would like to especially acknowledge my father, Dave McDonald, who I've been getting to know after too many years of absence.

Finally I want to acknowledge the love and friendship of my partner, Ingrid Rockel, who more than any other has had to bear the cost of this work, supporting me throughout and putting her own needs on hold to help me to finish.

TABLE OF CONTENTS

FIGURES AND TABLES

FIGURES:

TABLES:

ABSTRACT

Cysteine biosynthesis is an essential biosynthetic activity of *Escherichia coli*, as it is the sole pathway for synthesis of reduced sulphur. In this study a mutant, AM77, was found to be a cysteine requiring bradytroph. AM77 had previously been thought to be defective in osmoregulatory trehalose synthesis because of a mini Tn*10* insertion in a gene designated *otsC*. The mini Tn*10* insertion causing AM77's cysteine requirement was shown by complementation and sequencing to be within *cysQ*, a gene required for cysteine biosynthesis and which is immediately to 5' of the putative *otsC* insertion site. Another eight independently isolated osmosensitive mutants were also found to contain lesions in *cysQ*, but their phenotypes differed depending on the position of the mini Tn*10* insertion and on whether they contained secondary suppressor mutations. No evidence was obtained to support a role for AM77 in osmoregulatory trehalose synthesis, with all mutant phenotypes reversed by the addition of cysteine or other forms of reduced sulphur. The *cysQ* gene product is a putative phosphatase, and a member of a family of phosphatases which are inhibited by monovalent cations, specifically lithium. In this study it was shown that AM77 had a partially active CysQ protein, the activity of which was inhibited by the addition of NaCl. These results suggested that inhibition of the *cysQ* product might be a major cause of halosensitivity in *E. coli*. The transcriptional regulation of *cysQ* was characterised, and it was found that *cysQ* was activated by entry into stationary phase. Activation was at least partially modulated by the stationary phase σ factor, σ^s. Transcription of *cysQ* was also found to be affected by anaerobiosis, osmolarity and sulphur source. The observations in this study support the previously hypothesised role for *cysQ* function as a mediator of PAPS, a toxic intermediate in cysteine biosynthesis. In combination with the evidence for stationary phase activation of the *cysQ* gene, the hypothesis is presented that as well as being required for modulation of cysteine biosynthesis during growth, *cysQ* has a second role as a "switch", enabling rapid suppression of cysteine biosynthesis during stationary phase, possibly in response to carbon availability.

Notes on nomenclature

Genetic nomenclature

In this study all genes are referred to by standard bacterial genetic nomenclature :

gene: *cysQ*
gene product: CysQ

Where genes are designated, for example *cysQ*, this refers to a mutant allele unless otherwise indicated. Active alleles are designated as such, for example *cysQ* $^{+}$.

Wild type

Throughout this thesis wild type is used to designate W3110, the parent strain on which these experiments were based.

Abbreviations

Abbreviations used are listed in appendix IV.

Transposons

Mini Tn*10* transposons were named according to the nomenclature of Kleckner *et al.* (1991).

CHAPTER ONE

INTRODUCTION

This project was a continuation of work begun by A McLellan, a previous worker in this laboratory. The original aim of the project was to identify genes essential for osmoadaptation in *Escherichia coli*. A number of putative osmosensitive mutants were isolated, of which one, AM77, was analysed further. AM77 was characterised as being osmosensitive because of a deficiency in osmoregulatory trehalose synthesis. For this reason the locus disrupted in AM77 was designated *otsC*, (for **O**smoregulatory **T**rehalose **S**ynthesis **C**). The mutation was mapped to 95.7 minutes on the *E. coli* chromosome, and a complementing plasmid, pAD300, was constructed which reversed the mutant phenotype. Mutagenesis of pAD300 further defined the region necessary for *otsC* function to a 1.4kb sequence immediately to 3' of a gene, *amtA*, which was thought to be necessary for nitrogen metabolism. At this point I took over this project, with the initial aim of confirming the involvement of AM77 in osmoregulatory trehalose synthesis, followed by characterising the structure and regulation of the *otsC* gene, and determining the role of the *otsC* gene product in trehalose synthesis. In fact it was found in this study that the mutation in AM77 was in a gene required for cysteine biosynthesis, and subsequently that this gene, *cysQ* , is transcriptionally activated in response to the onset of stationary phase. Because osmoregulation, cysteine biosynthesis and response to starvation have all been shown to be implicated in the function of the *cysQ* gene I have attempted to review the features of each system which are of relevance to this project.

1. OSMOREGULATORY MECHANISMS OF *E. COLI*

Bacteria such as *E. coli* must be able to survive in a wide range of osmotic environments, from the colon and urinary tract to fresh water. Although not halophilic, *E. coli* has a variety of strategies for coping with these osmolarity changes. Adaptation is important because *E. coli* must maintain positive turgor pressure, an intracellular osmotic pressure higher than that of the outside environment, which has been suggested to provide the driving force for cell growth (Koch 1982). As the cell is freely permeable to water, changes in the medium osmolarity cause water influx or efflux, and consequently changes in turgor pressure. Hyperosmotic stress results in cytoplasmic water efflux, which decreases intracellular water activity, causing loss of turgor pressure and plasmolysis (Csonka 1989), and resulting in inhibition of cellular processes (Yancey *et al.* 1982, Arakawa and Timasheff 1985, Csonka 1989). Recent work by Cayley *et al.*(1991,1992) suggests that growth ceases when free cytoplasmic water (V_F - that which is not bound to macromolecules), decreases to zero, and that V_F is the fundamental

determinant of growth rate in osmotically stressed cells. Bound cytoplasmic water (V_B is not affected.

To cope with hyperosmotic shock *E. coli* must possess osmoregulatory mechanism (reviewed in Ingraham 1987, Csonka 1989), which are based on uptake or synthesis o solutes to increase the intracellular osmolarity and thereby increase intracellular wate activity. There are two stages to osmotic adaptation of *E. coli*, the primary respons being uptake of potassium ions (reviewed in Epstein 1986) and synthesis of counter ion such as glutamate, with a consequent increase in intracellular osmolarity and reversal o plasmolysis. The secondary response is synthesis or uptake of compatible solute which substantially replace potassium glutamate (Dinnbier *et al.* 1988, Welsh *et al.* 1991 Cayley *et al.* 1992).

The secondary response to osmotic shock , synthesis or uptake of compatible solutes, i the osmoregulatory mechanism of relevance to this study. Compatible solutes, fo example trehalose or glycine betaine, are so called because they do not inhibit enzymati function, whereas potassium ions inhibit gene expression and enzyme function *in vitr* (Csonka 1989, Booth and Higgins 1990). However, Cayley *et al.* (1991,1992) sugges the primary function of compatible solutes is to maintain V_F. They suggest tha potassium ions are replaced by compatible solutes (also called osmoprotectants), no because of the deleterious effects of potassium on enzymes, but because potassium ion are not excluded from V_B, whereas compatible solutes are. Consequently the compatibl solutes are present in the V_F (volume of cytoplasmic free water), the osmolarity of whic increases, thereby increasing turgor pressure.

In the absence of exogenous sources, *E. coli* synthesises trehalose as a compatibl solute (reviewed below). However, the most effective osmoprotectants are imported vi active transport systems, therefore, trehalose synthesis is repressed if externa osmoprotectants are available (Rod *et al.* 1988, Cayley *et al.* 1992). A wide range o compounds have been characterised as osmoprotectants including proline, glycine betain (reviewed in Csonka 1989), glutathione (McLaggan *et al.* 1990), ectoine (Jebbar *et al* 1992) and taurine (McLaggen and Epstein 1991). Glycine betaine can also b synthesised endogenously from choline by the Bet system (Eshoo 1988).

The osmoregulatory mechanism of *E. coli* constitutes a stimulon (Smith and Neidhar 1983), with a set of different genetic responses occurring to the one stimulus. There ha

systems such as heat shock. The primary regulatory mechanism is turgor pressure, which regulates potassium uptake (Csonka 1989), trehalose synthesis, and glycine betaine uptake (through direct action of potassium glutamate on the transcription transcription complex (Booth and Higgins 1990)).

2. TREHALOSE METABOLISM

Trehalose is a non-reducing disaccharide of glucose which is synthesised in *E. coli* and in many other organisms, in response to stresses such as osmotic shock, stationary phase heat shock, and dessication (Kaasen *et al.* 1992, Strøm and Kaasen 1993). In addition, when supplied exogenously, trehalose is utilised as a carbon source.

2.1. Trehalose in stress response

Trehalose has been found to be the most common organic osmolyte in osmotically stressed *E. coli*. Trehalose acts as the primary compatible solute, and it, glutamate and potassium are the only osmotically active solutes present in significant quantities in *E. coli*, in the absence of exogenous osmoprotectants such as glycine betaine (Larsen *et al.* 1987, Dinnbier *et al.* 1988, Welsh *et al.* 1991, Cayley *et al.* 1992). In the presence of glycine betaine, trehalose is unnecessary and synthesis is repressed (Rod *et al.* 1988, Cayley *et al.* 1992). Trehalose accumulation results from endogenous synthesis and is necessary for osmotolerance (Rod *et al.* 1988, Glæver *et al.* 1988); exogenous trehalose does not act as an osmoprotectant (Glæver *et al.* 1988). Accumulation of trehalose is induced by all non penetrant osmolytes, but not by penetrant solutes such as glycerol, suggesting that synthesis is induced by change in turgor pressure rather than by change in osmolarity (Welsh *et al.* 1991). Trehalose synthesis is a secondary response to osmotic upshock, following potassium uptake and glutamate synthesis. Accumulation starts within 30 minutes and trehalose has replaced glutamate as the major organic osmolyte within two hours of osmotic shock (Dinnbier *et al.* 1988). It has been suggested that potassium ions and glutamate are one of the control mechanisms for trehalose synthesis, as they increase the activity of trehalose-6-phosphate synthase *in vitro* (Glæver *et al.* 1988).

2.2. Trehalose biosynthesis

E. coli synthesises trehalose by a pathway common to many biological systems. Glucose-6-phosphate and UDP-glucose are converted to trehalose-6-phosphate by trehalose-6-phosphate synthase. Trehalose-6-phosphate is then hydrolysed to trehalose by trehalose-6-phosphate synthase. To date, four genes have been implicated in biosynthesis, these being *galU* (Rod *et al.* 1988, Glæver *et al.* 1988), *otsA* and *otsB* (Glæver *et al.* 1988) and *rpoS* (Kaasen *et al.* 1992). Mutations in any of these genes cause osmosensitivity, which is reversed by the addition of glycine betaine (Glæver *et al.* 1988). *GalU* encodes glucose-1-phosphate-UTP-pyrophosphorylase, which is required for synthesis of UDP-glucose, *otsA* and *otsB* are the structural genes for trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase respectively, and *rpoS* encodes σ_S, the stationary phase σ factor (Tanaka *et al.* 1993).

OtsA and *otsB* constitute an operon which maps to 42 minutes (Kaasen *et al.* 1992). The *otsBA* operon has low level constitutive activity, and is activated by increased osmolarity, which causes a five-fold increase in transcription (Glæver *et al.* 1988), and by stationary phase, which induces *otsBA* 8-10 times (Hengge-Aronis *et al.* 1991). Trehalose is also important in stationary phase heat shock and H_2O_2 resistance in *E. coli* (Hengge-Aronis *et al.* 1991) and *otsB* has been identified as the pex (or Post EXponential) gene, *pexA* (Hendrickson and Rudd 1992). Induction of *otsBA* is mediated by *rpoS* (Kaasen *et al.* 1992, Hengge-Aronis *et al.* 1991), the stationary phase σ factor, although low level osmotic induction occurs even in *rpoS*$^-$ strains (Kaasen *et al.* 1992). Transcription of *rpoS* is increased by osmotic stress (Hengge-Aronis *et al.* 1991).

Regulation of trehalose biosynthesis is by a futile cycle, with overproduction of trehalose, excretion of any excess, followed by TreA (the periplasmic trehalase) mediated recovery of glucose (Styrvold *et al.* 1991). *TreA* is osmotically regulated, exhibiting ten times induction upon osmotic upshock (Gutierrez *et al.* 1989, Repoila and Guteirrez 1991), and is also induced by *rpoS* (Hengge-Aronis *et al.* 1991).

2.3. Trehalose catabolism

Trehalose is able to be used as a carbon source by *E. coli*, which has two catabolic mechanisms; in high osmolarity trehalose is degraded by the periplasmic trehalase

encoded by *treA* (Boos *et al.* 1990), while in the absence of osmotic stress catabolism occurs via an osmotically repressed system encoded by *treB*, *treC* and *treE* (Boos *et al.* 1990). Posphorylated trehalose is imported by the phosphotransferase system, and the phosphate is removed. Trehalose is then hydrolysed to glucose by amylotrehalase (*treC*), in a UDP-glucose dependent reaction (absent in *galU* strains) (Boos *et al.* 1990). *TreB* and *treC* map to 96.5 minutes on the *E. coli* linkage map, but are not part of the same operon (Boos *et al.* 1990).

3. CHARACTERISATION OF *OTSC*

3.1. Introduction

A previous worker in this laboratory, Alex McLellan, mapped and cloned a locus, *otsC*, thought to be involved in osmoregulatory trehalose synthesis. McLellan was interested in finding uncharacterised genes involved in osmoregulation, and his strategy was to create osmosensitive mutants by transposon mutagenesis, with selection for loss of growth on minimal media in the presence of a normally permissible concentration of NaCl. Strain W3110 was selected as the parent strain. W3110 is a derivative of *E. coli* K12, and has been used for physical mapping of the *E. coli* chromosome (Kohara *et al.* 1987). The use of W3110 for the physical map is especially important as Kohara generated a set of λ clones containing overlapping regions of genomic DNA, which enable rapid mapping of chromosomal insertions, and provides both a means of transducing mutations between strains (Kulakauskas *et al.* 1991) and a source of wild type DNA for cloning.

3.2. Isolation of AM77

Mutagenesis was achieved by use of mini Tn*10kan* (Way *et al.* 1984), which are both smaller and more stable than Tn*10*, as the transposase gene is placed outside the transposon. Transductants were selected that grew on M63 minimal media, but not on M63 with 0.35M NaCl added. Because of the slow growth of W3110 on M63, transductants were scored for growth after two days incubation. Eighteen osmosensitive mutants were isolated and of these nine were shown by Southern hybridisation to have transposon insertions in what appeared to be a common 2.9kb *EcoRI* fragment. McLellan chose one of these nine mutants, AM77, to characterise further. The transposon insert was cloned into pBR322 by selection for kanamycin resistance, resulting in plasmid pAD77 (see figure 5.2). The insertion was physically mapped by

location of the insertion position by comparison to the physical map of Kohara *et al.* (1987). The insertion location was confirmed by hybridisation to Kohara's ordered library of λ phages. Both methods placed the transposon insertion of AM77 at 95.7 minutes on the *E. coli* genomic map.

3.3. Phenotype of AM77

AM77 was osmosensitive in liquid media, with OD_{600} being at wild type levels after 2 days in minimal media alone, whereas addition of varying concentrations of NaCl retarded growth compared with W3110. Osmosensitivity could be transduced to other strains by Phage P1, segregating with the kanamycin resistance marker. AM77 was also sensitive to glucose free minimal medias; AM77 failed to grow on M63 with trehalose, or trehalose plus glycerol, as the carbon source(s), or with trehalose plus 0.25M NaCl. Growth of AM77 was also poor on galactose plus glycerol compared with W3110. McLellan concluded that trehalose must exert a toxic effect in an *otsC* background. McLellan's assertion that AM77 was defective in osmoregulatory trehalose synthesis was based on the observed osmotolerance and also on observations of the intracellular trehalose levels. These results are reprinted (see table 1.1 below). Because of overproduction of glycogen in W3110 (Rod *et al.* 1988), intracellular trehalose could not be measured by the anthrone method. Instead, McLellan employed a method based on HPLC (high performance liquid chromotography) separation of sugars and other polyols, followed by quantification by reaction with potassium permanganate, giving oxidation of the permanganate to manganate with a visible spectrum shift (McLellan 1992). Transduction of *otsC::*Tn*10kan* into a *galU* strain (deficient in osmoregulatory trehalose synthesis) resulted in a strain, 12XU, that was even more osmosensitive than either parent. McLellan interpreted this as evidence for *otsC* being a pleiotrophic gene required for functions other than osmoregulatory trehalose synthesis.

3.4. Complementation and mapping

AM77 was complemented by a 13kb *BamHI* fragment from the complementary Kohara phage λ K656 cloned into the low copy plasmid pJEL109 (Larsen unpublished data), to give pAD109, and by a *SalI-HindIII* subclone of this fragment in pBR322 (pAD300). pAD300 was able to fully complement AM77 for growth on M63 plus 0.35M NaCl and on trehalose plus glycerol medias. An *EcoRI* subclone from the 13kb *BamHI* fragment, corresponding to that into which *otsC::*Tn*10kan* originally inserted, was unable to complement AM77.

The physical region necessary for complementation of AM77 was mapped by McLellan using mini Tn*10cam* mutagenesis (Kleckner *et al.* 1991). Ten insertions were isolated, with eight of these clustered around AM77's insertion site, and mapping either just within, or just below, a recently identified gene designated *amtA* (Fabiny *et al.* 1991). AM77 was found to grow as wild type on M9 media with limiting nitrogen, confirming AM77 to be *amtA*$^+$. The remaining two insertions mapped to a position about 1.2kb to 3' of the *amtA* gene. From these results, along with the position and orientation of two Tn*10-LK* translational gene fusions, McLellan concluded that *otsC* was positioned adjacent to the 3' end of *amtA*, and was transcribed clockwise on the *E. coli* chromosome. On the basis of these insertion positions, McLellan suggested that *otsC* might be a putative reading frame identified in the *amtA* sequence, which is 214 bp below *amtA*, and which extends for 89 bp to the end of the published sequence (Fabiny *et al.* 1991).

Table 1.1. Trehalose content of cells
(mg trehalose/10^9)

Strain	M63 + 0M NaCl	M63 + 0.4M NaCl
W3110	0	3.0
AM77	0	1.2
AM77 pAD200	0	9.0
AM77 pAD300	0	3.5

(reprinted from McLellan 1992)

3.5. β-galactosidase activity

Two mini Tn*10-LK* translational fusion derivatives of pAD300 were obtained by McLellan. Neither of these fusions, pKL8 or pKL10, complemented AM77 for growth on M63 + 0.35M NaCl. Both fusions were mapped to just below the 3' end of *amtA* and both fusions had β-galactosidase activity, although pKL8's activity was greater. McLellan examined the expression of pKL8 in M63. He found that gene

activity was decreased by the addition of trehalose but not by glycine betaine, although this effect was only observed in low osmolarity (half strength M63). He also observed that adding NaCl increased activity in a concentration dependent manner up to 0.1M, but that concentrations above this level decreased activity. In a *pcnB* strain, which gives low copy number of colE1-based plasmids (Lopalito *et al.* 1986), pKL8's expression was decreased 160 fold. In addition expression was found to increase in a concentration-dependent manner upon addition of NaCl, to a maximum when 0.6M NaCl was added.

4. *CYSQ* AND *AMTA*

The mini Tn*10* insertion of AM77 was originally mapped to below *amtA*, a gene originally thought to be necessary for ammonium methylammonium uptake (Amt) (Jayakumar *et al.* 1989), but subsequently found to be involved in cysteine biosynthesis, and renamed *cysQ* (Neuwald *et al.* 1992). During the course of this study it became apparent that in fact the insertion was in *cysQ*, therefore cysteine biosynthesis and *cysQ* are reviewed below.

AmtA was originally identified by mutations that failed to grow on minimal media with 100mM ammonium acetate as the sole nitrogen source, but that were able to grow on minimal media in the presence of 20mM ammonium. The insertion was mapped to 95.8 minutes on the *E. coli* chromosome (Jayakumar *et al.* 1989), and mutants were found to be complemented by a plasmid containing a 3.4kb *SalI-HindIII* fragment, subclones of which were sequenced to give the *amtA* sequence (Fabiny *et al.* 1991). *AmtA* had a 246 codon open reading frame (ORF), corresponding to a protein of about 27kD, as well as a putative Shine and Delgarno sequence and promoter, and a simple inverted repeat sequence that could act as a transcriptional terminator. The encoded protein appeared to be cytoplasmic, as it lacked signal sequences for protein export, and *phoA* mutagenesis isolated no insertions in *amtA*, although *phoA* insertions were isolated from the neighbouring gene, *cpdB* (Jayakumar *et al.* 1989).

Simultaneously, Douglas Berg's group (Neuwald *et al.* 1992) had isolated a cysteine auxotroph later characterised as *cysQ*. The auxotrophy was incomplete, with slow growth occurring, thus *cysQ* mutants could be considered to be bradytrophs, or slow growers. When grown on defined media *cysQ* mutants formed single colonies after three days, however, in anaerobic conditions they grew as wild type. Single colonies

recultured after growth in minimal conditions showed the same growth rate as their parent, confirming the mutation was leaky, rather than highly revertible. A variety of insertion positions, and a deletion of the whole promoter and the 5' end of *cysQ*, all gave the same leaky phenotype when transduced into the chromosome in single copy. Leakiness was found to be strain dependent, with some strains giving barely perceptible growth. Neuwald *et al.* (1992) found *cysQ* to be identical to *amtA*, and that addition of sulphite or cysteine restored growth to both *cysQ* and *amtA* mutants in conditions of limiting nitrogen. They suggested that as neither sulphite nor cysteine can be used as nitrogen sources, this was evidence for *amtA*'s growth deficiency being due to partial nitrogen starvation, combined with partial cysteine starvation caused by the *cysQ* mutation.

Growth of *cysQ* mutants was also found to be restored by sulphite or thiosulphate, and mutants had normal sulphate transport, ATP sulphurylase and APS kinase activities (Neuwald *et al.* 1992). CysQ (the putative *cysQ* product) was not involved in modulation of *cpdB*, the gene for cyclic phosphodiesterase, which borders *cysQ*. Nor was it the pyrophosphatase required for efficient APS synthesis. These results narrowed the range of action of CysQ to cysteine biosynthesis, between APS synthesis and PAPS reduction.

Neuwald and coworkers (1992) sequenced *cysQ* and observed there to be only 17bp between the -35 regions of *cysQ* and *cpdB*, with an apparent CAP site overlapping the *cysQ* promoter and oriented to activate *cpdB*. They found no obvious CysB binding site. In addition they detected a second promoter within *cysQ* and oriented towards *cpdB*, which was transcribed *in vivo*.

5. CYSTEINE BIOSYNTHESIS

Cysteine biosynthesis is a fundamental biosynthetic pathway, which is necessary not just to provide cysteine for protein synthesis, but also as the assimilatory pathway for all reduced sulphur in *E. coli* . Reduced sulphur is required for methionine biosynthesis, as well as for the synthesis of many other essential cofactors and cellular components, such as biotin, glutathione and spermidine. The basic biochemistry of the cysteine biosynthetic pathway of *E. coli* and *Salmonella typhimurium* has been characterised (reviewed by Kredich 1987). The pathway is summarised in figure 1.1, while a list of genes implicated in cysteine biosynthesis is presented in table 1.2.

Table 1.2. Genes involved in cysteine biosynthesis

Gene	Location (min)	Cysteine regulon	Activity
cysA	52	+	Sulphate premease
cysB	28	+	Positive regulatory protein
cysC	59	+	APS kinase
cysD	59	+	ATP sulphurylase
cysE	80	+	Serine transacetylase
cysG	73	-	Sulphite reductase sirohaem synthesis
cysH	59	+	PAPS reductase
cysI	59	+	Sulphite reductase haemoprotein
cysJ	59	+	Sulphite reductase flavoprotein
cysK	52	+	*O*-Acetylserine (thiol)-lyase-A
cysM	52	+	*O*-Acetylserine (thiol)-lyase-B
cysN	59	+	ATP sulphurylase GTPase
cysP	52	+	Thiosulphate binding protein
cysQ	96	?	PAPS phosphatase?
cysS	12	?	Cysteinyl tRNA ligase
cysT	52	+	Sulphate permease
cysX	80	?	Regulatory?
cysW	52	+	Sulphate permease
cysZ	52	?	Sulphate transport
sbp	89	+	Sulphate binding protein
?	?	+	L-Cystine transport: CTS-1
grx	19	?	Glutaredoxin
trxA	86	?	Thioredoxin

(compiled from information in Mark *et al.* 1977, Leyh *et al.* 1992, Tei *et al.* 1990, Kredich 1987, Kren *et al.* 1988, Sirko *et al.* 1990)

There are two convergent components to cysteine biosynthesis, sulphide synthesis and O-Acetyl serine (OAS) synthesis. Sulphide is the form of reduced sulphur directly utilised to synthesise cysteine, while OAS provides cysteine's carbon backbone. The entry point into the cysteine biosynthetic pathway is determined by the sulphur source, with more reduced forms of sulphur being utilised preferentially. For the purposes of this study, the important aspect of cysteine biosynthesis is the pathway for reduction of sulphate to sulphide, while other aspects of sulphur metabolism are only mentioned where they are of relevance. The complete sulphur assimilation pathway is controlled as a regulon (the cysteine regulon), with activation by a transactivator protein, CysB. Allosteric control is important for induction and anti-induction of CysB, and for repression of OAS synthesis.

5.1. Transport

Sulphate uptake occurs via a sulphate permease, encoded by *cysA*, *cysT* and *cysW*, which has functional homology to permeases such as those for histidine and maltose (Sirko *et al.* 1990). The sulphate permease also requires sulphate and thiosulphate binding proteins (*sbp* (Hellinga and Evans 1985) and *cysP* (Sirko *et al.* 1990)). *CysA*, *cysT* and *cysW* are encoded in an operon at 52 minutes on the *E. coli* chromosome (Hryniewicz *et al.* 1990)

5.2. Sulphate activation

The initial step in sulphate reduction is activation by ATP sulphurylase to adenosine 5'-posphosulphate (APS), followed by further phosphorylation by APS kinase to 3'-adenosine 5'-phosphosulphate (PAPS). The initial phosphorylation by ATP sulphurylase is extremely energetically unfavourable, and is driven by hydrolysis of pyrophosphate and removal of APS by phosphorylation to PAPS (Kredich 1987). The activities of both ATP sulphurylase and APS kinase decrease rapidly upon slowing of growth, suggesting enzyme degradation is occurring. By contrast, *O*-Acetylserine (thiol)-lyase-A and sulphite reductase are unaffected by growth phase (Kredich 1987). Genes involved in sulphate activation are *cysD*, *cysN* and *cysC*, which are transcribed as the *cysDNC* operon under control of the cysteine regulon (Leyh *et al.* 1992). ATP sulphurylase is encoded by *cysD*, which is the structural gene, and *cysN*, which is an intrinsic GTPase (Leyh *et al.* 1992). *CysC* is the structural gene for APS kinase.

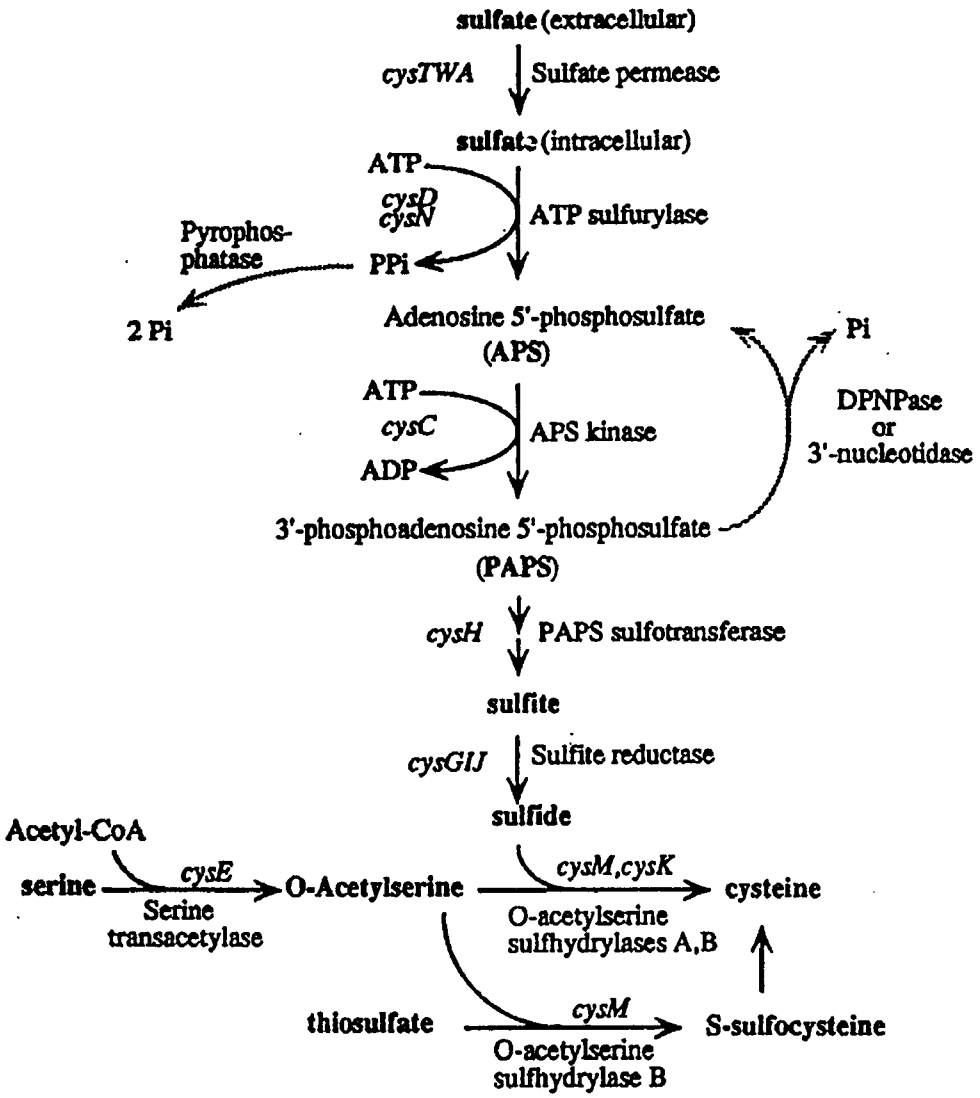


Figure 1.1. The pathway for cysteine biosynthesis in *E. coli* (Derived from information contained in Kredich (1987) and Neuwald *et al.*(1992)).

5.3. PAPS reduction

PAPS reduction occurs in two steps, the first being the reduction of PAPS reductase (*cysH*) by thioredoxin, followed by the reduction of PAPS to PAP plus free sulphite (Krone *et al.* 1991). In addition reduced thioredoxin must be regenerated, which may occur via thioredoxin reductase, as occurs in *Saccharomyces cerevisiae* (Schwenn *et al.* 1988). Glutaredoxin can also act as the reductant of PAPS reductase, as *trxA* (deficient in thioredoxin) or *grx* (deficient in glutaredoxin) mutants are prototrophic, whereas *grx trxA* double mutants are cysteine auxotrophs (Russel *et al.* 1990). The PAPS reductase gene is transcribed as part of the *cysJIH* operon, which is at 59 minutes on the *E. coli* chromosome, but separate from *cysDNC* (Krone *et al.* 1991, Ostrowski *et al.* 1989).

5.4. PAPS toxicity

Mutants of *cysH* in *S. typhimurium* were unstable during storage on plates, but not in long-term liquid cultures, accumulating secondary mutations in earlier genes in cysteine biosynthesis, which increased viability (Gillespie *et al.* 1968). *TrxA grx* double mutants of *E. coli* were similarly unstable, with all *trxA grx* double mutants obtained by Russel *et al.* (1990) having secondary mutations, primarily in the *cysA* locus. Supplying cysteine, but not casamino acids, prevented these secondary mutations occurring, as did a *cysC* or *cysA* background. The secondary mutations were supplemented by sulphite and cystine, and interestingly also by methionine (see chapter 4 section 4). The results of Gillespie *et al.* (1968) and Russel *et al.* (1990), suggested either PAPS, or a derivative of PAPS, to be toxic, resulting in mutants that were blocked in PAPS reductase activity, and that accumulated suppressor mutations in earlier cysteine biosynthetic genes to prevent PAPS accumulation. It is suggested by Neuwald and coworkers (1992), that it is in the modulation of PAPS that *cysQ* has its role in cysteine biosynthesis. They further suggest that CysQ, the *cysQ* product, might either be required for PAPS reductase activity, be involved in sequestering PAPS, or act as a phosphatase (based on its homology to inositol monophosphatase (see below). *CysQ* mutants were observed to be unstable on defined media and, as with *cysH* and *grx trxA* mutants, they developed suppressor mutations in earlier cysteine biosynthetic genes, especially in those encoding the sulphate permease, for example *cysA*.

5.5. Sulphite reduction to sulphide

The final step in sulphide formation is a 6 electron reduction of sulphite to sulphide, which is catalysed by sulphite reductase, an enzyme which contains a novel haem group, sirohaem, which is also necessary for nitrite reductase and cobalamin synthesis. Free sulphide is released, as the product of sulphate reduction.

5.6. *O*-Acetylserine and cysteine synthesis

Both *O*-Acetylserine (OAS) and cysteine synthesis are catalysed by an enzyme complex which contains serine transacetylase and *O*-Acetylserine sulfhydrylase-A. Serine transacetylase catalyses synthesis of OAS from serine and acetyl coenzyme A, and is encoded by *cysE* (Tei *et al.* 1990). *O*-Acetylserine (thiol)-lyase-A, encoded by *cysK*, catalyses the formation of cysteine and is present either in complex with serine transacetylase, or free in the cytoplasm. *E. coli* also contains a second *O*-Acetylserine (thiol)-lyase, B, which is required for incorporation of thiosulphate by reaction with OAS to form S-sulphocysteine.

5.7. Utilisation of different sulphur sources

E. coli is able to utilise a wide range of reduced and oxidised sulphur sources, which includes sulphate, sulphite, sulphide, thiosulphate, cystine, cysteine, cystathione and djenkolic acid, with each of these having different entry points into the sulphur assimilatory pathway (Kredich 1987). Recently it was found that a number of sulphonates, such as taurine and cysteate, could also act as sulphur sources for *E. coli*, supporting growth to the same levels as sulphate (Uria-Nickelsen *et al.* 1993). Methionine cannot act as a sulphur source, although its presence has a sparing effect on cysteine biosynthesis (Qureshi *et al.* 1975, Kredich 1987). This is because *E. coli* lacks the enzymes to allow the regeneration of reduced sulphur from methionine.

As well as determining the entry point into cysteine biosynthesis, the sulphur source chosen for use in the laboratory has a number of experimental implications, some of which I will set out below. Many reduced forms of sulphur are unstable in oxidising conditions. For example, cysteine spontaneously oxidises to cystine above pH2 in a reaction catalysed by metal ions (Postgate 1963, Kredich 1987). Sulphide is rapidly hydrolysed to H_2S at pH7. Sulphite is extremely rapidly oxidised to sulphate; when aerated at physiological pH7 its half life at 37°C is approximately 75 minutes

(Postgate 1963). Cystine, sulphate and thiosulphate are largely stable. Another important effect is that cysteine inhibits growth in minimal conditions, due to inhibition of threonine deaminase (Harris 1981, Sorensen and Pederson 1991). This inhibition causes transient delays in growth due to amino acid starvation, even with cysteine concentrations as low as 0.04μg/ml. Harris (1981) reported this starvation to be reversed by the addition of isoleucine and threonine, but Sorensen and Pederson (1991) found only partial reversal of growth retardation.

5.8. Cym mutations

Cym mutations are a class of mutations of cysteine biosynthetic genes of *S. typhimurium* described by Qureshi *et al.* (1975). Cym mutants were complemented, not just by addition of cysteine, but also to wild type growth levels by methionine. Cym mutants were not able to convert methionine to cysteine and Qureshi and coworkers concluded that enzyme activity assays were inconsistent with the effect being caused by cysteine sparing. Mutants were obtained in *cysD*, *cysG*, *cysA*, *cysC*, *cysH*, *cysI* and *cysJ*, with all mutations occurring between, or at the ends of, genes. The phenotypical characteristics observed were a growth lag with cysteine of 1-3 hours, but no growth lag for methionine, with both cysteine and methionine restoring growth, in contrast to known cysteine and methionine mutants. Within the cym mutants there were several phenotypes, which differed in the extent of response to cysteine and methionine. Finally, it was found that the presence of methionine enabled mutants to accumulate sulphur from sulphate; in other words, methionine was either stimulating the cysteine biosynthetic enzymes, or allowing cysteine biosynthesis to occur.

5.9. Anaerobic cysteine biosynthesis

Little has been published about anaerobic sulphur metabolism in *E. coli*, but some basic facts are known about *S. typhimurium*. Mutants of sulphite reductase or of *cysG* of *E. coli* are auxotrophs in anaerobic conditions, whereas only *cysG* mutants are auxotrophic in *S. typhimurium*. This suggests *S. typhimurium* has a second, anaerobic, sirohaem containing sulphite reductase which is absent or defective in *E. coli* (Huang and Barrett 1990). *S. typhimurium* is also able to synthesise sulphide anaerobically from thiosulphate via a *cysM* requiring pathway, with *cysM* mutants being bradytrophs (Hallenback *et al.* 1989). Neither anaerobic sulphide synthesis pathway is controlled by the cysteine regulon, nor are they inhibited by cysteine (Hallenback *et al.* 1989). The relevance of these pathways to *E. coli* is unknown.

5.10. Regulation of cysteine biosynthesis

Cysteine biosynthesis is co-ordinately controlled as the cysteine regulon, with three levels of regulation: transcriptional control by CysB; induction and anti-induction of CysB function; and feedback inhibition of OAS synthesis. CysB, which activates genes involved in cysteine biosynthesis, is a member of the LysR family of transcriptional activators. CysB requires the presence of OAS or N-acetyl serine (NAS) for activation (Ostrowski and Kredich 1989), while sulphur shortage is also required for activation, as thiosulphate and sulphite act as anti inducers (Hryniewicz and Kredich 1991, Ostrowski and Kredich 1990). In addition, feedback inhibition of serine transacetylase by either sulphide or cysteine regulates the amount of OAS present, thereby controlling CysB activity and the cysteine regulon (Kredich 1987).

6. PROTEINS HOMOLOGOUS TO CYSQ

CysQ, the *cysQ* gene product, shares homology with a family of proteins, of which those functionally characterised are all phosphatases, with phosphorylated sugar substrates. The most well characterised member of the family is inositol monophosphatase (IMPase), therefore I will refer to the proteins collectively as the IMPase family. A protein homology data base search (Altschul *et al.* 1990), found more than ten proteins or putative proteins which, on the basis of their amino acid sequences, were members of the IMPase family. They have been isolated from a wide range of evolutionarily distinct species, and in loci of very different function. These include reading frames within the *trpE* locus of the eubacteria *Leptospira biflexa*, the quinic acid utilisation cluster of *Neurospora crassa* and *Aspergillus nidulans*, the streptomycin locus of *Streptomyces griseus*, and the exopolysaccharide locus of *Rhizobium leguminosarum* (Yelton and Peng 1989, Geever *et al.* 1989, Hawkins *et al.* 1988, Borthakur *et al.* 1988, Pissowotzki *et al.* 1991).

The enzymatic function of three IMPase family members have been determined; all three are phosphatases that act on phosphorylated sugar substrates. Both IMPase and inositol polyphosphate 1-phosphatase (IPPase) are involved in the inositol signalling pathway of eukaryotes (McAllistar *et al.* 1992), while fructose-1,6-bisphosphatase (F-1,6-BPase) is a key regulatory enzyme of gluconeogenesis (Zhang *et al.* 1993). Of the other reading frames SuhB, the product of the *suhB* gene of *E. coli*, is known to be involved in modulating stability of mRNA for the heat shock σ factor, σ_{32}

(Yano *et al.* 1990, Nagai *et al.* 1991). *S. typhimurium* also has a *cysQ* gene, on the basis of sequence data from the neighbouring *cpdB* gene. The first 98 amino acids of an open reading frame, in the correct position and reading frame, and with 88% amino acid homology, are visible in the published sequence (Liu *et al.* 1990). Another gene, *HAL2*, with a putative product homologous to the IMPase family, has recently been identified in *S. cerevisiae* (Gläser *et al.* 1993). *HAL2* was identified as a gene which conferred increased halotolerance when present in high copy number, and is identical to *MET22*. The addition of methionine, but not cysteine, proline or asparagine, was found to increase the halotolerance of *S. cerevisiae*. *HAL2* mutants are methionine auxotrophs. The pathway for sulphur assimilation in *S. cerevisiae* is somewhat different to that of *E. coli*, with methionine as the primary product of biosynthesis, and cysteine biosynthesis a reversible side reaction. Consequently *S. cerevisiae* sulphur uptake mutants have MET designations, although the initial reduction pathway from sulphate to sulphide is identical to that of *E. coli* (Cherest and Surdin-Kerjan 1992). Thus, the *HAL2* gene product, HAL2, may be directly homologous in function to CysQ. The amino acid sequences of CysQ, IMPase, *S. typhimurium* CysQ, SuhB and HAL2 are compared in figure 5.5.

6.1. Structure of IMPase family

The IMPase family has common features at the level of amino acid sequence, and those that have been characterised have common enzymatic characterisitics and conformational structures. The three proteins to which enzymatic actions have been ascribed, IPPase, IMPase and F-1,6-BPase, all require Mg^{2+} ions for activity, although F-1,6-BPase can also utilise two Mn^{2+} or Zn^{2+} (Zhang *et al.* 1993). All are non-competitively inhibited by Li^{+} ions (Majerus 1992, Zhang *et al.* 1993) and IMPase is inactivated by phenylglyoxal, an arginine specific reagent (Majerus 1992). Ganzhorn *et al.* (1993) found IMPase to be extremely heat stable, despite there being no disulphide bonding. At a structural level the crystal structures of both IMPase and F-1,6-BPase have been solved (Bone *et al.* 1992, Zhang *et al.* 1993). Monomers of either have very similar $\alpha\beta\alpha\beta\alpha$ conformational structures, although F-1,6-BPase has an additional α helix, involved in allosteric inhibition by AMP. F-1,6-BPase is active as a tetramer, while IMPase is dimeric, with the active sites of both located in a hydrophilic cleft, which is close to the dimer junction (Zhang *et al.* 1993). Conserved residues that are required for metal binding, primarily aspartate and glutamate, but including an arginine residue that may also be involved in dimer formation, are indicated in figure 5.5. At the sequence level there are some obvious similarities between homologues, but also noteable differences. Except in its active

sites, F-1,6-BPase has very poor homology to IMPase and other members of the family, which makes its conformational and functional similarities the more remarkable. The primary level of sequence similarity occurs in three consensus sequences, each of which contains residues necessary for metal binding in F-1,6-BPase. These consensus sequences are indicated in figure 5.5. IPPase also shares little homology to either IMPase or F-1,6-BPase, except in residues required for the active site.

7. POTENTIAL REGULATORY MECHANISMS OF *CYSQ*

As previously stated, the mini Tn*10* insertion from AM77 was found in this study to be in *cysQ*. As a gene necessary for cysteine biosynthesis, the logical regulatory system for *cysQ* would appear to be transactivation by CysB, the cysteine regulon transactivator. Neuwald *et al.* (1992) had, however, observed *cysQ* to lack CysB consensus binding sequences, which suggested that different transcriptional control mechanisms might be operating for *cysQ*. In addition, it is has become increasingly apparent that many genes are regulated by a number of different regulatory genes, and in response to a number of stimuli. Because of the gene expression characteristics observed in this study, the effects of a number of other regulatory systems were examined. It was found that RpoS, and possibly CAP, but not supercoiling are implicated in the regulation of *cysQ*. These are reviewed below.

7.1. Supercoiling

Control of supercoiling has been implicated in the transcriptional regulation of a range of genes in response to enviromental stimuli, specifically anaerobiosis and osmotic stress, both of which are of interest in the present study. Both anaerobiosis and increased osmolarity cause increased negative supercoiling. Genes known to be regulated by supercoiling include *proU* and *recA* (Ní Bhriain *et al.* 1989, Wang and Syvanen 1992). It is thought that supercoiling affects σ^{70} promoters, which can be twist sensitive. The *proU* promoter, for example, is underwound due to the spacing between its -10 and -35 regions. Increased potassium concentration is thought to increase negative supercoiling, thereby causing activation of the promoter (Wang and Syvanen 1992). The degree of supercoiling in the cell is normally regulated by interactions between gyrase and topoisomerase enzymes, which increase and decrease negative supercoiling respectively. Experimentally the effects of supercoiling on gene expression can be examined in gyrase$^-$ strains, or by the addition of the gyrase inhibitors naladixic acid or novobiocin. An example of a supercoiling regulated

biosynthetic promoter is that of the *his* operon, which is derepressed by entry into stationary phase or by novobiocin, and repressed by anaerobiosis and increased osmolarity (O'Byrne *et al.* 1992).

7.2. CAP activation

CAP (catabolic gene activator protein, also called CRP or cAMP receptor protein) activation is the mechanism by which *E. coli* activates various inducible catabolic pathways for utilisation of alternative carbon sources. CAP is a transcriptional activator that is induced by increased cAMP levels (Brickman *et al.* 1973), and that activates the promoters of genes such as those for lactose, galactose and maltose utilisation. It also activates *cpdB*, the divergently-transcribed gene bordering *cysQ*, although this activation is relatively weak (Liu and Beacham 1990). There is a potential CAP binding site capable of activating *cpdB* above the -35 region of the *cpdB* promoter. CAP is a member of the helix-turn-helix class of DNA binding proteins, and binds as a dimer to a 22 bp consensus sequence. Binding is associated with bending of DNA, and activation with protein-protein interactions between CAP and RNA polymerase (Ebright 1993).

7.3. Aerobic and anaerobic gene expresssion

CysQ is only required during aerobic growth (Neuwald *et al.* 1992). In *E. coli* ArcA and ArcB are transcriptional repressors of aerobic genes in anaerobic conditions. They are members of the two component sensor-regulator family, with ArcA the regulator (reviewed in Lin and Iuchi 1991).

7.4. RpoS, the stationary phase σ factor (σ^S)

RpoS (also called KatF) was originally isolated as a pleiotrophic transcriptional regulator implicated in the control of a wide range of genetic loci including microcins B17 and C7, *glgS* (glycogen synthesis), *ots* (osmoregulatory trehalose synthesis), *katE* (catalase) and *bolA* (a "morphogene") (Hernández-Chico *et al.* 1986, Diáz-Guerra *et al.* 1989, Hengge-Aronis and Fischer 1992, Hengge-Aronis *et al.* 1991, Loewen and Triggs 1984, Lange and Hengge-Aronis 1991). Sequencing of the structural gene, *rpoS* (*katF*), showed it to have homology to the σ^{70} family of σ factors (Mulvey and Loewen 1989). More recently Tanaka *et al.* (1993) have proved RpoS to have σ factor activity *in vitro*. RpoS is regulated in response to growth phase, with induction of *rpoS* upon entry into stationary phase (Lange and Hengge-

Aronis 1991). RpoS is a central regulator of starvation and stationary phase, with *rpoS* mutants being deficient in, for example, at least 32 carbon starvation proteins (McCann *et al.* 1991), as well as in the phenotypes listed above. Stationary phase survival of *rpoS* strains is also greatly impaired during both nitrogen and carbon starvation (McCann *et al.* 1991, Lange and Hengge-Aronis 1991). For this reason RpoS has been called σ^S, the stationary phase σ factor (Lange and Hengge-Aronis 1991). As well as being induced by stationary phase, σ^S is induced by osmotic shock (Hengge-Aronis *et al.* 1993). As a result at least 18 other genes are induced by σ^S in response to osmotic stress. Expression of *rpoS* is regulated post-transcriptionally, with transcriptional fusions to *rpoS* being expressed at increasing levels through exponential growth, but translational fusions showing little activity until stationary phase (McCann *et al.* 1993, Loewen *et al.* 1993). In addition, *rpoS* is autoregulated (McCann *et al.* 1993).

The specificity of the promoter sites which σ^S recognises is still being elucidated. Regine Hengge-Aronis (in press) has observed a lack of strong σ^{70} promoters before σ^S regulated genes. Tanaka *et al.* (1993) observed σ^S to recognise both σ^{70} and σ^S promoters *in vitro*, and postulated that there were three groups of promoters: those recognised only by σ^{70}, those recognised by both σ^{70} and σ^S, and those recognised only by σ^S. The function of these promoters correlated with the specificity of recognition observed. For example, purely stationary phase genes were only transcribed by σ^S. Tanaka and coworkers (1993), therefore, suggested a model whereby gene expression is determined by an equilibrium between σ^{70} and σ^S levels, with the equilibrium moving towards σ^S in stationary phase.

8. RESPONSE OF *E. COLI* TO STARVATION

Bacteria have primarily been studied in rich media, but in nature the norm is for long periods of starvation with brief periods of nutritional plenty. Thus, bacteria must be able to survive these periods of starvation yet rapidly return to growth when nutrients become available. *E. coli* is a non-differentiating bacteria that does not form resistant structures, such as endospores (Matin *et al.* 1989). Despite this *E. coli* can remain viable in starvation conditions for periods of a year or more (Tormo *et al.* 1990).

The response of *E. coli* to starvation entails production of of 90 or more new proteins, which have been characterised for conditions including phosphorus, nitrogen and carbon shortage. These new proteins are either specific to starvation for

a particular nutrient, or common to a number of starvation conditions. Matin (1991), identified 55 proteins induced by carbon starvation and 47 induced by nitrogen shortage, of which 24 were common to both responses. The overlapping groups of proteins produced in response to a particular stimulus, such as starvation, has led to the concept of stimulons, a group of genes induced by a common stimulus and involved in a common response (Smith and Neidhart 1983). The changes in protein expression in response to starvation were accompanied by morphological changes, which included decreased cytoplasmic volume, increased periplasmic volume, changed cell surface properties and cell wall composition (Siegele and Kolter 1992). In response to starvation, resistance of *E. coli* to a range of stress conditions, including osmotic shock, oxidative stress and heat shock also increases (Siegele and Kolter 1992).

There are two levels of starvation response. The first is where in response to a shortage of a particular essential nutrient, bacteria manufacture enzymes required to concentrate that nutrient from the media. Examples of this are the responses to limiting nitrogen, phosphorus or carbon. The second is when the media becomes exhausted for a particular nutrient, at which stage the bacterium enters stationary phase (Matin 1991).

Two sets of genes with different functions are induced by starvation: genes to escape from stationary phase and genes involved in stationary phase resistance. These differ in both their function and regulation. The starvation genes are not required for long term survival, or stationary phase resistance (Matin 1991). Their role is in allowing escape from stationary phase by satisfying the cells requirement for a particular nutrient. The survival genes, by contrast, are not required for short term survival, but cells which are defective in survival genes have decreased long term viability (Matin 1991).

8.1. Starvation proteins

Starvation proteins are expressed in response to shortage of particular nutrients and are involved in obtaining that missing, essential, nutrient, thereby enabling escape from starvation. The primary responses characterised have been those for carbon, phosphorus and nitrogen starvation, all of which result in synthesis of specific sets of proteins. Carbon starvation causes activation of *cst* genes (Matin 1991) which are regulated by CAP protein, while both phosphorus and nitrogen utilise two component

sensor/regulator systems (Matin *et al.* 1989). To my knowledge, nothing has been published on the induction of proteins in response to sulphur starvation.

8.2. Resistance proteins

The resistance proteins are the so-called pex (or post exponential) proteins, which include most of the core proteins induced in response to all starvation conditions (Matin 1991). Pex proteins are required for stationary phase survival. Their function is to make the cell more resistant, thereby preparing in advance for the potential stresses that might be encountered during starvation, as a rapid response to stress in the form of the synthesis of new proteins is not possible. Pex proteins are independent of induction by specific nutrient response transactivators such as CAP, but many are also induced by other stress situations, including heat shock, oxidative stress and osmotic stress (Matin 1991). Pex proteins have a wide range of functions including protein folding, protein degradation, cell wall components, osmoprotectant synthesis (trehalose), protection from oxidative stress (catalase), and synthesis of energy storage molecules (glycogen) (Matin *et al.* 1989, Hengge-Aronis *et al.* 1991). Other genes are beginning to be characterised, in both *E. coli* and *S. typhimurium*, which are required specifically for survival (Tormo *et al.* 1990, Spector and Cubitt 1992).

Most pex genes are transcriptionally activated by σ^S, which has been suggested to be a second major σ factor, other than σ^{70} (Hengge-Aronis 1993). *RpoS* mutants, deficient in σ^S, are defective in stationary phase survival (Siegele and Kolter 1992), as well as in other resistances mediated by the pex proteins, for example stationary phase heat shock (McCann *et al.* 1991, Matin 1991). Some pex proteins are also induced in response to minor σ factors, such as σ^H, the heat shock σ factor (Matin 1991), while others have been observed to be under the negative control of CAP (Spector and Cubitt 1992).

9. EXPERIMENTAL OBJECTIVES

The primary aim of this study was molecular characterisation of the putative *otsC* locus, along with determining whether there were other loci of related function in the region. There were three initial experimental objectives: characterisation of the mini Tn*10* insertion point of AM77 and determination of the minimum sequence required for complementation of AM77; clarification of the phenotype of AM77, specifically

to confirm whether otsC was required for osmoregulatory trehalose synthesis; and examining the regulation of the *otsC* gene, thereby allowing comparison to the other more well known osmoregulatory genes.

With the finding that the mutation in AM77 was in fact in *cysQ* the focus of this study shifted, although the basic experimental approach was retained. The emphasis became clarifying the relationship between *cysQ* and osmosensitivity, and determining the genetic control of *cysQ*, as this had not been characterised. In practice the general experimental aims remained very similar.

CHAPTER TWO

METHODS

1. BACTERIAL STRAINS, BACTERIOPHAGES AND PLASMIDS

Bacterial strains, bacteriophages and plasmids used in this study are listed in Table 2.1.

2. BUFFERS AND MEDIA.

Buffers and solutions used in this study are described in Appendix 1. Media used in this study are listed in Appendix 2. To minimise fluctuations between individual cultures during growth assays in M63 or M9 media, the media were prepared in larger volumes with all common ingredients, such as antibiotics, added to the larger volume. Aliquots were removed for individual cultures, and other supplements were then added.

2.1. Antibiotics

Antibiotics were added to agar plates or overnight liquid cultures to the following concentrations.

Ampicillin	(Amp)	100μg/ml
Chloramphenicol	(Cam)	30μg/ml
Kanamycin	(Kan)	50μg/ml
Nalidixic acid	(Nal)	30μg/ml
Streptomycin	(Str)	50μg/ml
Tetracycline	(Tet)	15μg/ml

2.2. Supplements

Supplements were added to plates or liquid cultures to the following concentrations:

Isopropyl-β-D-thio-galactopyranoside	(IPTG)	0.5mM

5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside	(X-Gal)	30 μg/ml
glycine betaine		1.0 mM
choline		1.0 mM
potassium sulphite		0.3 mM
potassium thiosulphate		0.3 mM
cysteine		0.3 mM
methionine		0.3 mM

All other amino acids are listed in appendix 3.

Table 2.1. Bacterial Strains, Bacteriophages and Plasmids.

Strain, phage and plasmids	**Genotype**	**Reference or source**
Bacteria *Escherichia Coli*		
71-18	*supE thiD*(*lac-proAB*) F'(*proAB*+ *lacI*^{q} *lacZ* Δ M15)	Yansich -Perron *et al.*
DH5α	*supE44 hsdR17 recA1endA1 gyrA96 thi-1 relA1*	D. Hanahan
W3110	F$^-$ *rpsL* IN(*rrnD-rrnE*)1	B. Bachmann
ZK592	*arg, ara, his, leu, pro, recB21, recC22, sbcB15, thr, thi, rpsL, Δ(lac-proAB), endA, sbc-15, hspR4,* (F' *traD36, proAB, lacI*$_Q$, *ZΔM15*)	Winkler
ER1647	F$^-$ *fhuA2* Δ *(lacZ)r1 supE44, trp31, hisI, metB1, mcrA1272*::Tn*10*, Δ(*mcrC-mrr*)*102*::Tn*10*, *xyl-7, mtl-2, rpsL104, recD1014*	B. Bachmann
SP850	Hfr, λ$^-$, e14$^-$, *relA1, spoT1,* Δ(*cya-1400*)*::kan, thi-1* Hfr	Shah and Peterkofsky
NK7402	F$^-$*trpB83*::Tn*10*, λ$^-$, IN(*rrnD-rrnE*)1,	N. Kleckner
N3002	F$^-$, λ$^-$, *cys-95*::Tn*10*, IN(*rrnD-rrnE*)1,	R.G. Lloyd

UM56-64	F⁻, *ara, leuB, azi, fhuA, lacY, proC, tsx, purE, supE, galK*, λ⁻, *trpE, xthA, his, rfbD, mgl, katF, argG, rpsL, glpR, xyl, mtl, ilvA, katG, metA, thi*	P. Loewen
CB64	F⁻, *trp-75, cysB93, tfr-8*	M. Jones-Mortimer
KL166	Hfr, λ⁻, *gyrA13, relA1, thyA24, spoT1, thi-1, deoB13*	K.B. Low
AM77	W3110 *cysQ77::*Tn*10kan*	A. McLellan
AD36	W3110 *cysQ36::*Tn*10kan*	A. McLellan
A26	W3110 *cysQ26::*Tn*10kan*	A. McLellan
H8	W3110 *cysQ8::*Tn*10kan*	A. McLellan
L16	W3110 *cysQ16::*Tn*10kan*	A. McLellan
K41	W3110 *cysQ41::*Tn*10kan*	A. McLellan
T4	W3110 *cysQ4::*Tn*10kan*	A. McLellan
V62	W3110 *cysQ62::*Tn*10kan*	A. McLellan
V77	W3110 *cysQV77::*Tn*10kan*	A. McLellan
71CH7	71-18 *CH7::*Tn*10cam*	This study
71KL8	71-18 *cysQ8::*Tn*10-LK*	This study
71KL10	71-18 *cysQ10::*Tn*10-LK*	This study
WCH7	W3110 *CH7::*Tn*10cam*	This study
WKL8	W3110 *cysQ8::*Tn*10-LK*	This study
WKL10	W3110 *cysQ10::*Tn*10-LK*	This study
MM1	ER1647 *cysQ8::*Tn*10-LK*	This study
MM2	ZK592 *cysQ10::*Tn*10-LK*	This study
MM3	ZK592 *CH7::*Tn*10cam*	This study
MM5	CB64 *trpB83::*Tn*10*	This study
MM6	WCH7 *cysQ77::*Tn*10kan*	This study
MM7	71KL8 *cysB93 trpB::*Tn*10*	This study
MM8	71KL8 *cysC::*Tn*10*	This study
MM9	71KL8 *katF3*	This study
MM10	71KL8 *gyrA13*	This study
MM16	W3110 *cysQ16::*Tn*10-LK*	This study
MM17	W3110 *cysQ41::*Tn*10-LK*	This study
MM18	71KL8 *CH7::*Tn*10cam*	This study
MM19	71KL10 *CH7::*Tn*10cam*	This study

plasmids		
pBR322	Amp^r, Tet^r	Bolivar *et al.*
pUC19	Amp^r	Yanisch-Perron *et al.*
pUC18	Amp^r	
pAD300	Amp^r *cysQ* $^+$	A. McLellan
pAD109	Amp^r *cdpB*$^+$ *cysQ* $^+$	A. McLellan
pAD822	Cam^r *cysQ* $^+$	A. McLellan
pAD77	Kan^r Tet^r Amp^r *cysQ77::*Tn*10kan*	A. McLellan
pKL8	Amp^r Kan^r *cysQ8::*Tn*10-LK*	A. McLellan
pKL10	Amp^r Kan^r *cysQ10::*Tn*10-LK*	A. McLellan
pCH7	Amp^r Cam^r *CH7::*Tn*10cam*	A. McLellan
pMM77	Amp^r *cysQ77::*Tn*10*	This study
pMM80	Amp^r *cysQ8::*Tn*10 lacZ*$^+$	This study
pMM81	Amp^r *cysQ8::*Tn*10 lacZ*$^+$	This study
pMM100	Amp^r *cysQ10::*Tn*10 lacZ*$^+$	This study
pMM101	Amp^r *cysQ10::*Tn*10 lacZ*$^+$	This study
pMM300	Amp^r *cysQ*$^+$	This study
pMM305	Amp^r *cysQ*$^-$	This study
pMM310	Amp^r *cysQ*$^+$	This study
pMM373	Amp^r *cysQ*$^+$	This study
pMM406	Amp^r *cysQ*$^+$	This study
pMM410	Amp^r *cysQ*$^+$	This study
pMM450	Amp^r *cysQ*$^-$	This study
phages		
λK446		Kohara *et al.* 1987
λK656		Kohara *et al.* 1987
$P1_{KC}$		Pittard

3. BACTERIOLOGICAL METHODS

Purification of single colonies, toothpicking, and determination of antibiotic resistance were all done by standard bacteriological techniques. *E. coli* were always incubated at 37°C. Overnight cultures were prepared by inoculation of a single, well-isolated, bacterial colony into 3ml LB with antibiotics where appropriate. Cultures were grown overnight at 37°C with aeration by shaking.

Exponential cultures were obtained by 1/50 or 1/100 dilution of a bacterial overnight into LB (+ appropriate antibiotics). For determinations of growth in defined media, overnight cultures were pelleted and resuspended twice in 1 x M63 or M9 salts (as appropriate). Fresh cultures were inoculated to 1/100, 1/50, or 1/20 dilutions, depending on the condition being determined. All determinations were the average of 2 or 3 independent cultures.

Growth of liquid cultures was determined by measuring the turbidity at 600 nm (OD_{600}) in an LKB Ultrospec spectrophotometer with cell sipper. Samples were compared to a blank of the appropriate media. If OD_{600} was greater than 0.6, appropriate dilutions were made, as at above 0.6 optical density is not proportional to cell numbers. Absorbance readings of 1.0 were found to equate to a viable cell count of approximately 7×10^9 cell forming units/ml (cfu/ml).

Bacterial strains were kept at -80°C in storage media. Stocks for day-to-day use were streaked for single colonies on antibiotic plates, which were kept for no longer than 2-3 weeks.

Anaerobic growth assays were performed by adding the appropriate media to sterile test tubes, which, after inoculation, were sealed with parafilm to exclude oxygen, and incubated without shaking at 37°C. Similar conditions have been shown to cause activation of anaerobically induced genes (Volkert 1989).

To test for auxotrophy sterile 25mm filters were placed in the centre of M63 plates and the strains of *E. coli* to be tested were streaked in 3 lines, parallel to the edge of the filter, and at different distances from it. 200µl of the stock amino acid to be tested (at 100 times working concentration) was dripped onto the filter. Plates were incubated at 37°C and scored for supplementation of the bacterial streaks by diffused amino acid.

Viable cell counts were determined by spotting aliquots of a serial dilution of a culture on to TB plates. Single colonies were counted after overnight growth.

To determine whether antibiotic markers and auxotrophy were maintained in minimal conditions, a loop of culture was streaked for single colonies on a non selective plate (LB). Single colonies were then streaked on antibiotic plates to check whether antibiotic markers were retained, and on minimal plates to see whether auxotrophic suppressor mutations had occurred.

4. DNA MANIPULATION AND CLONING TECHNIQUES

Agarose gel electrophoresis, phenol/chloroform extractions, restriction enzyme digestion, alkaline phosphatase treatment and ligation of DNA were all carried out according to the methods of Sambrook *et al.* (1989), or according to the manufacturers instructions. For restriction digestions involving two restriction endonucleases requiring different buffer conditions, the buffer most suitable for both was used. If this was not possible, the DNA was digested by one enzyme, precipitated, and then digested with the other enzyme.

All centrifugation steps involving DNA preparation were performed at 12000 rpm for 5 minutes at 4°C unless otherwise stated. All cells were pelleted by centrifugation at 6000 rpm for 3 minutes at 4°C.

4.1. Elution of DNA by centrifugation (Heery *et al.* 1990)

The required DNA band was excised from an agarose gel. The gel slice was placed in a punctured eppendorf tube plugged with siliconised glass wool, which was inserted into another eppendorf tube. The DNA was separated from the agar by centrifugation (2 minutes, 6000 rpm, 4°C), and the DNA in the bottom eppendorf was precipitated. After pelleting by centrifugation, the DNA was dissolved in TE buffer, and the yield of the eluted band estimated by gel electrophoresis.

4.2. Filling in of overhanging 5' DNA ends (Sambrook *et al.* 1989)

DNA digested by a restriction endonuclease was precipitated and redissolved in 10µl of sterile distilled H_2O. 1µl of 2mM dNTP solution (dATP, dCTP,

dGTP, dTTP), 2.5µl of 10x blunt end buffer, 11.5µl of H_20 and 1µl of DNA polymerase 1 (Klenow fragment) was added. The solution was then incubated at 25°C for 30 minutes. This procedure filled in the cohesive ends of restricted DNA. Prior to ligation DNA Polymerase 1 was heat killed at 70°C for 5 minutes.

4.3. Precipitation of DNA (Sambrook *et al.* 1989)

All precipitation of DNA was by Alcohol precipitation. 1/10 volume of 3M sodium acetate and 2 volumes of ethanol were added to the DNA solution. The DNA was left for 10 minutes to several hours at -20°C, and then pelleted by centrifugation. After washing with 70% ethanol and drying, the DNA was resuspended in water or TE.

4.4. *Bal31* Digestion (Sambrook *et al.* 1989)

DNA prepared by the LiCl method was completely digested by a restriction endonuclease. To determine the amount of *Bal31* required serial twofold dilutions of *Bal31* in 1 x *Bal31* buffer were added to 9µl aliquots of a solution of:

4µl	linearised DNA
48µl	dH_2O
13µl	5 x *Bal31* buffer.

All aliquots were then incubated at 37°C for 30 minutes and the digestion was stopped by the addition of 1µl of 200mM EGTA, after which the degree of digestion was determined by analysis on an agarose gel. The enzyme concentration yielding fragments of 2000-6000bp in 30 minutes (the desired size range) was selected for preparation of DNA for subcloning.

To prepare *Bal31* digested DNA for subcloning, 60µl of linearised DNA (20µg) was added to 226µl dH_2O, 65µl 5 x *Bal31* buffer and 10µl *Bal31* (15 units). 45µl aliquots were incubated at 37°C for 2, 4, 6, 8, 10, 12, 14, and 16 minutes, and reactions were stopped with 5µl 200mM EGTA. The extent of *Bal31* digestion was confirmed by analysis on an agarose gel. The DNA was extracted with phenol/chloroform and then precipitated and resuspended in 20µl of TE buffer.

5. PREPARATION OF DNA

5.1. Alkaline extraction of plasmid DNA (Derived from Birnboim and Doly (1979) by Kennedy (1988)).

An 1.5ml aliquot of bacterial overnight culture was poured into an eppendorf tube and the cells were pelleted by centrifugation. The supernatant was removed and the pellet resuspended in 150µl of Solution I. After a five minute incubation at room temperature, 200µl of Solution II was added and mixed gently until the solution became viscous and clear. After a five minute incubation on ice 150µl of Solution III was added and the tube mixed thoroughly to break up the precipitate. The tube was incubated on ice for five minutes, the precipitate pelleted by centrifugation and the supernatant poured off into a new tube.

One ml of 100% ethanol was added to the supernatent and DNA was allowed to precipitate at room temperature for 5 minutes. The DNA was pelleted by spinning and then washed once with 70% ethanol. After drying, the DNA pellet was suspended in 30µl TE buffer.

5.2. Boiling preparation of plasmid DNA (Holmes and Quigley 1981).

1.5ml of an overnight culture was pelleted in an eppendorf and resuspended in 200µl of cold STET buffer. 16µl of fresh 10mg/ml lysozyme was added and the tube was boiled for 90 seconds. The tube was spun and the supernatent decanted into a fresh eppendorf. 160µl of cold isopropanol was added and DNA was precipitated by 20 minutes incubation at -20°C. DNA was pelleted by centrifugation, washed with 70% ethanol, then resuspended in 30µl TE after drying. The resuspended DNA was incubated at 65°C for 15 minutes to ensure denaturation of endonucleases.

5.3. Plasmid DNA preparation - lithium chloride method (A. Fellowes, personal communication).

100ml of overnight culture was pelleted by centrifugation (4000 rpm, 10 minute, 4°C) in a 250ml Sorval tube. The pellet was resuspended in 5ml of Solution I, followed by 10ml of Solution II. The tube was gently rolled until the solution became viscous and clear. After 5 minutes at room temperature, 5ml of Solution III was added and mixed. The precipitate was pelleted by centrifugation (10000 rpm, 20 minutes, 4°C) and the supernatant strained into a fresh tube.

An equal volume of cold isopropanol was added and the tube incubated on ice for 10 minutes. The DNA was pelleted by centrifugation (2500 rpm, 10 minutes, 4°C) and the pellet was dissolved in 1ml of TE. An equal volume of 5M LiCl was added, and incubated for 10 minutes on ice. The precipitate was pelleted by centrifugation (2500 rpm, 10 minutes, 4°C) and the supernatant transferred into a clean tube. An equal volume of cold isopropanol was added and the tube incubated on ice for 10 minutes. The DNA was pelleted by centrifugation (2500 rpm, 10 minutes, 4°C), dissolved in 0.4ml of TE and transferred to an eppendorf tube. 10µl of RNase A (10mg/ml) was added and incubated for 15 minutes at 37°C. 500µl of 2.5M NaCl-20% PEG, was added and the tube incubated on ice for 10 minutes. The DNA was pelleted by centrifugation and redissolved in 250µl of TE.

After phenol/chloroform extraction the DNA was precipitated and the pellet washed in 1ml of 70% ethanol. After drying, the pellet was dissolved in 200µl TE buffer.

5.4. Chromosomal DNA preparation

1.5 ml of an overnight culture was pelleted and washed in 300µl TES, then repelleted. Pellets were resuspended in 100µl of 25% sucrose in TE, and 20µl of 5mg/ml lysozyme and 40µl of 0.25M EDTA were added. The solution was mixed gently then incubated at 37°C for 15 minutes. The following solutions were then added and mixed: 10µl 20% SDS; 5µl 1 mg/ml RNaseA (in 0.1M sodium acetate); 200µl dH_2O. The tube was then incubated at 37°C for a further 30 minutes to lyse the cells. 5µl of 10 mg/ml pronase was added before a further incubation of 1 hour at 37°C. The solution was extracted twice with

phenol/chloroform, and once with chloroform. DNA was precipitated from the aqueous phase, washed with 70% ethanol, and resuspended in 200µl TE.

6. TRANSFORMATION METHODS

6.1. Calcium chloride method (Sambrook *et al.* 1989).

6.1.a. Preparation of competent cells.

Exponentially grown cells were pelleted by centrifugation (6000 rpm, 5 minutes, 4°C) and the supernatant removed. The cells were suspended in 1/2 the original volume of cold Solution I and incubated on ice. After 20 minutes the cells were pelleted by centrifugation, the supernatant removed and the pellet resuspended in 1/4 the original volume of cold Solution II. The cells were incubated on ice for 20 minutes, pelleted and then resuspended in 1/15 of the original volume of cold Solution II. After 30 minutes on ice, 200µl aliquots of cells were used immediately, or stored at -80°C after the addition of 7µl of 99.5% dimethyl sulfoxide (DMSO).

6.1.b. Transformation.

100-200ng of DNA was added to a 200µl aliquot of competent cells and the solution was gently mixed. The mixture was left on ice for 30 minutes, and then incubated at 42°C for 2 minutes, and then on ice for a further 2 minutes. 1ml of LB broth was added and the cells were incubated at 37°C for one hour. The cells were pelleted, resuspended in 200µl of LB broth and spread on a selective plate.

6.2. Electroporation (Dower *et al.* 1988).

6.2.a. Preparation of Competent Cells

A 50 ml exponential culture was grown to an OD600 of 0.5-0.8, and chilled on ice for 30 minutes. Cells were pelleted by centrifugation, suspended in 50ml dH_2O and incubated on ice for 30 minutes. The centrifugation was repeated, the pellet suspended in 25ml dH_2O and incubated on ice for a further 30 minutes. The cells were pelleted (4000 rpm, 15 minutes, 4°C) and the pellet resuspended in 1ml of ice cold 10% glycerol. After a 30 minute incubation on ice and a final centrifugation step, the pellet was suspended in 200µl of ice cold

10% glycerol. Aliquots of 40µl were either used immediately or stored at -80°C until required.

6.2.b. Electroporation

An aliquot of competent cells was thawed on ice. Sterile electroporation cuvettes (0.2cm gap) and the chamber slide were also chilled on ice. Electroporation was carried out using a Biorad Gene Pulser, set to 25µF and 250kV, with a resistance of 200 ohms.

Approximately 100ng of DNA was added to the cells, mixed, and left on ice for one minute. The mixture was transferred to the chilled cuvette and pulsed once. An 1ml aliquot of SOC media was added immediately and mixed with the sample, which was then transferred to an eppendorf tube. The cells were incubated in the eppendorf tube at 37°C for one hour. A 200µl aliquot was plated on a selective plate. The remaining cells were pelleted by centrifugation, resuspended in 200µl of LB and then spread on a selective plate.

To electroporate ligated DNA the ligation mix was precipitated and resuspended in 5µl dH_20. One µl of DNA was used per transformation (Zabrovsky *et al.* 1990).

7. TRANSDUCTION

7.1. P1 transduction (variation of Gowrishankar 1985).

7.1.a. Phage purification

100µl of an overnight culture of a susceptible host in LB + 2.5mM $CaCl_2$ was infected with 100µl of a 10^{-5} or 10^{-7} dilution of $P1_{KC}$ stocks. After adsorption at 37°C for 20 minutes the phage/bacteria mix was added to 1.5ml of 45°C molten top agar and poured onto a prewarmed LB plate. The plate was incubated at 37°C until single plaques appeared, and a single plaque was removed and resuspended in 100µl of LB.

7.1.b. Preparation of plate lysate

5µl of the resuspended single plaque was added to 100µl of an overnight solution of the host donor (grown in LB + 2.5mM $CaCl_2$). Phage were adsorbed for 15 minutes at 37°C. The bacteria were added to a top agar overlay

and poured onto an LB plate. The plate was incubated at 37°C until phage plaques just began to overlap. 5 ml of cold LB was poured over the plate, and left overnight at 4°C. The lysate was removed, chloroform was added to lyse any remaining cells, and the lysate spun to remove bacterial debris.

7.1.c. $P1_{KC}$ transduction

10^8 $P1_{KC}$ phage were added to 1ml of an overnight culture of recipient bacteria (in LB + 2.5mM $CaCl_2$), and incubated for 20 minutes at 37°C to allow adsorption. Cells were pelleted and washed in 0.1M citrate buffer, pelleted again and then resuspended in 1.5ml of LB + 2.5mM sodium citrate. Cells were then incubated for 1 hour at 37°C to express antibiotic markers. After elaboration cells were pelleted, and then resuspended in 200µl of 0.1M citrate buffer. Resuspended cells were spread on appropriate antibiotic plates for selection of transductants.

7.2. λ K656 transduction (a combination of the methods of Kulakauskas (1991) for λ transduction, and Gowrishankar (1985) for P1 transduction).

λ K656 transduction, preparation of plate lysates, and purification of single plaques were all by the same protocol as for $P1_{KC}$ (see section 7.1), with the exception that all bacterial cultures were mid exponential subcultures grown in LBMM.

8. ASSAYS OF β-GALACTOSIDASE (Miller 1972)

β-galactosidase activity of *lacZ* fusions was determined by measuring the conversion of *O*-nitrophenyl-β-d-galactoside (colourless) to galactose + *O*-nitrophenol (yellow), which can be measured by absorbance at 420nm.

Cultures were measured either at stationary phase or during exponential growth (following purification for at least 10 generations in exponential growth). All β-galactosidase assays were from at least duplicate cultures, and each determination of β-galactosidase activity was performed in duplicate.

Aliquots, sufficient for measurement of OD_{600} and β-galactosidase activity, were removed from cultures. 100µl (stationary) or 500µl of culture were added to bijou bottles containing 900µl or 500µl of Z buffer respectively. 50µl

chloroform and 25µl 0.1% SDS were added, and bottles were vortexed to lyse cells. Bottles were incubated for 5 minutes at 30°C then 200µl 4mg/ml ONPG (4mg/ml in 0.1M phosphate buffer) was added. Samples were incubated at 30°C until noticeable yellow colour resulted, at which point 500µl of 1M Na_2CO_3 was added to stop the β-galactosidase activity. With experience it was possible to stop reactions with an OD_{420} of 0.5-1.0, which was the optimum range for absorbance measurements. All samples were centrifuged to remove cell debris (2 minutes, 13000 rpm), and absorbance was measured at 420nm and 550nm in an LKB Ultrospec spectrophotometer with cell sipper. Absorbance of each sample was determined in triplicate.

β-galactosidase activity was determined by the formula of Miller (1972):

$$\text{Miller Units} = 1000 \times \frac{OD_{420} - (1.75 \times OD_{550})}{t \times v \times OD_{600}}$$

OD_{600} was measured at the time of the assay
t = the time of the reaction in minutes
v = the volume of culture used (µl)

9. POLYMERASE CHAIN REACTION

The polymerase chain reaction (PCR) was used to amplify fragments from single copy chromosomal sequences. A single bacterial colony (Krishnan *et al.* 1991) was resuspended in 10µl dH_2O and added to the following reaction mix:

10µl	10 x buffer
4µl	dNTP solution
2µl	primer 1
2µl	primer 2
68µl	dH_2O

and 60µl sterile mineral oil was added to prevent evaporation. Following an initial 5 minute denaturation at 94°C, 1U of Taq polymerase in 4µl 1 x Taq dilution buffer was added. The sample was then subjected to the following amplification:

30 cycles of

54°C	45 seconds
72°C	60 seconds
90°C	45 seconds

followed by a final 5 minute incubation at 54°C.

10. SEQUENCING

Sequencing of double stranded pUC18 based plasmids was done using a Pharmacia T7 sequencing kit and ^{35}S labelled ATP.

10.1. Denaturation of DNA and primer annealing

4µl of 2M NaOH (in 1mM EDTA) was added to 1µg DNA in 16µl dH_2O, and incubated at room temperature for 20 minutes. DNA was precipitated by addition of 14µl dH_2O, 6µl 3M sodium acetate and 120µl 100% ethanol. The solution was left overnight at -20°C, pelleted, washed in 70% ethanol and dried. The pellet was resuspended in 8.6µl dH_2O, 2µl annealing buffer, and 3.4µl primer (mini Tn*10-IS1OR*). After 20 minutes incubation at 37°C to allow annealing the DNA was left at room temperature, but placed on ice 5 minutes before use.

10.2. Labelling

A labelling mix of

1µl	^{35}S-ATP
3µl	labelling mix
2µl	T7 polymerase (at 1.5U/µl)

was added to the denatured DNA. The mix was incubated at room temperature for 5 minutes.

10.3. Termination

4.5µl of labelled DNA was added to each of 4 tubes containing 2.5µl of either A, C, G, or T termination mixes (prewarmed at 37°C). Termination reactions were incubated at 37°C for 5 minutes and 5µl of stop solution was then added to each tube.

10.4. Electrophoresis

An LKB 2010 Macrophor Sequencing System was used for sequencing gel electrophoresis. A 6% Polyacrylamide sequencing gel was prepared (Appendix I). The gel was prerun at 1200V for one hour. Sequencing reactions were placed at 85°C for 2 minutes, and then 3µl of each reaction mix was loaded. The gel was run for approximately 90 minutes at 1500V. The sequencing gel was washed twice in 10% Acetic acid for 30 minutes. It was then dried for 1-2 hours at 75°C. Amersham ^{35}S Sequencing Hyperpaper was exposed overnight, or as necessary, and autoradiographs were developed by standard techiniques.

CHAPTER THREE

RESULTS

1. PHENOTYPE OF AM77

1.1. Osmosensitivity of AM77

AM77 was originally isolated and characterised as an osmosensitive mutant deficient in osmoregulatory trehalose synthesis (McLellan 1992). Initially my aim was to confirm that AM77 was osmosensitive. AM77 failed to grow on M63 + 0.35M NaCl and growth was not restored by either the osmoprotectant glycine betaine or its precursor, choline. The growth of AM77 was substantially less than that of the parent strain W3110 even in the absence of added NaCl. On M63 plates large numbers of obvious suppressor mutations were observed, as outlined more fully in section 7. Colonies containing secondary mutations were visible as larger but heterogeneous colonies against a faint background of AM77 growth. As McLellan had observed unusual growth on M63 with trehalose as the carbon source, I also repeated these experiments, finding that AM77 did not grow, or grew poorly, with trehalose or trehalose and glycerol as the carbon source(s). Nor was growth observed with trehalose + 0.25M NaCl or on M9 media with limiting nitrogen. All results are summarised in figures 3.4 and 3.5.

1.2. Complementation

McLellan found AM77 to be complemented by pAD300, a plasmid containing a 3.4kb *SalI -HinDIII* fragment which included the complete coding sequence from *cysQ* and the 5' end of *cpdB* (figure 5.1). The *SalI-HinDIII* fragment was cloned from λ K656, one of an ordered set of λ clones generated by Kohara and co-workers (1987) and containing a 13kb *BamHI* fragment from 95.7 minutes on the *E. coli* chromosome. Two other plasmids, pAD109 (appendix 5.3) and pAD822, also complemented AM77. Both contained the complete 13kb *BamHI* fragment from λ K656, but cloned into different vectors; pAD109 was based on pJEL109, while pAD822 was a pACYC184 derivative. This study confirmed that pAD300, pAD109 and pAD822 all complemented AM77. It was also confirmed that pAD822 had a separate toxic effect which was proved to be caused by pACYC184, the presence of which retarded the growth of W3110 in minimal conditions. For this reason pAD822 was not used further in this study. To isolate the region of pAD300 required for complementation of AM77, two strategies were used; characterisation of previously generated non-complementing mutations of pAD300, and serial deletion and subcloning of the complementing *SalI-HinDIII* fragment.

Figure 3.1A. Insertion positions of mini Tn*10* mutants of pAD300. 3, 5, 6, 7, 9, 10, 21 and 22 all refer to the insertion positions of the pCH plasmids of those numbers, and were determined by restriction mapping. **8, 10** and **77** refer to the insertion positions of pKL8, pKL10 and AM77 respectively, which were determined by sequencing of the insertion junctions.

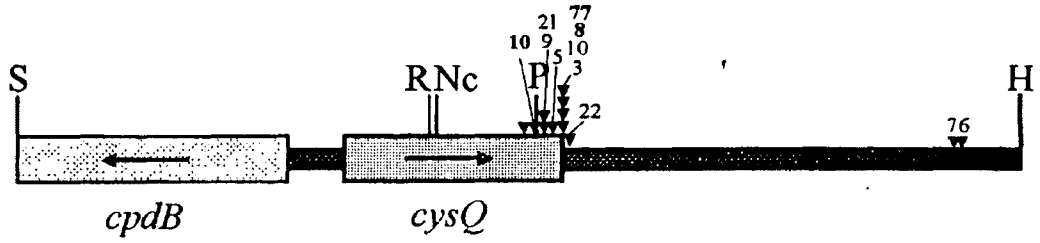


Figure 3.1B. Complementation of AM77 by mini Tn*10* mutants of pAD300."+" indicates complementation, "-" indicates lack of complementation.

	M63	M63 + 0.35M NaCl
pAD300	+	+
pAD109	+	+
pCH3	+	-
pCH5	-	-
pCH6	+	-
pCH7	+	-
pCH9	-	-
pCH10	+	-
pCH21	-	-
pCH22	+	-
pKL8	+	-
pKL10	-	-

Figure 3.2A Deletion subcloning of the complementing *SalI-HinDIII* fragment of pAD300. All pMM plasmids are based on pUC19 except pMM450, which is a pUC18 derivative. pMM300 is the complete *SalI-HinDIII* fragment, pMM305, 310, 373, 406 and 410 are *Bal31* deletion subclones, and pMM450 is a subcloned *EcoRI-HinDIII* fragment.

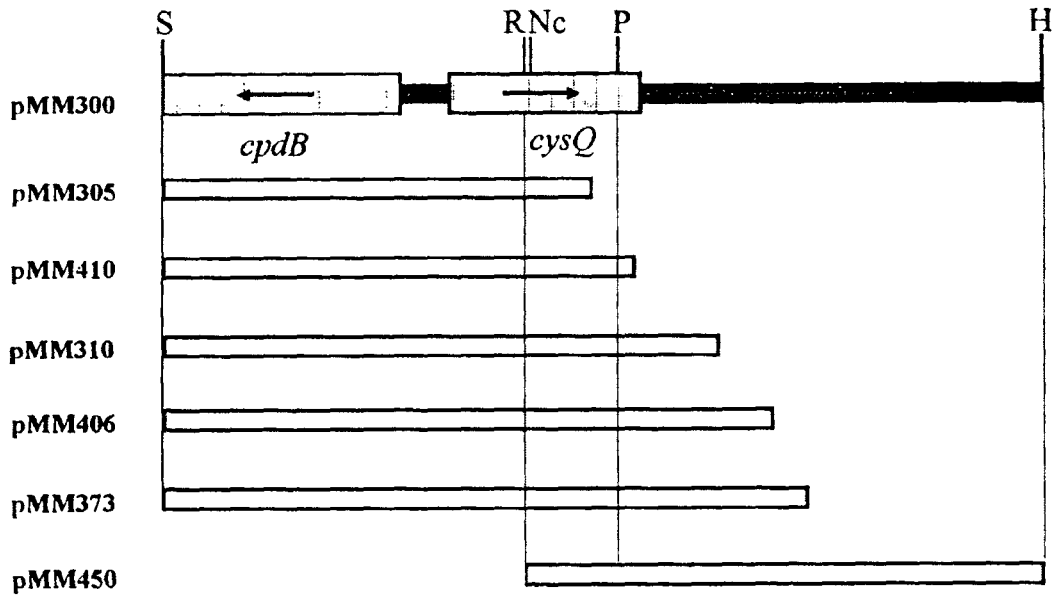


Figure 3.2B Complementation of AM77 and WKL10 by deletion subclones of the *SalI-HinDIII* fragment of pM300: "+" indicates complementation, and "-" lack of complementation.

	AM77						
	pMM300	pMM305	pMM410	pMM310	pMM406	pMM373	pMM450
M63	+	-	+	+	+	+	-
M63 + 0.35M NaCl	+	-	+	+	+	+	-

	WKL10			
	pMM300	pMM305	pMM410	pMM450
M63	+	-	+	-
M63 + 0.35M NaCl	+	-	+	-

1.2.a. Characterisation of non-complementing mutants of pAD300

Eight mini Tn*10cam* and two mini Tn*10-KL* mutants of pAD300 were generated by McLellan, all of which were classified as *otsC* on the basis of their failure to complement AM77 for growth on M63 + 0.35M NaCl. All ten plasmids were examined for their ability to restore growth to AM77 on M63 plates, with or without added 0.35M NaCl. None grew in the presence of added NaCl. On M63 alone there were two phenotypes; one group had approximately wild type growth, while the other group behaved like uncomplemented AM77. Complementation results are shown in figure 3.1B. To correlate these complementation results to insertion positions the transposon insertion sites were mapped by restriction digestion. Map positions of all insertions are shown in figure 3.1A and were determined by *EcoRI-BamHI* and *HinDIII-BamHI* double digests (pCH) or *EcoRI* and *PstI* digestion (pKL). The orientations of the two kan-lac fusions $cysQ_8$::Tn*10-LK* and $cysQ_{10}$::Tn*10-LK* were determined by the position of their internal *EcoRI* sites. Only approximate mapping of $cysQ_8$::Tn*10-LK* was possible because of the absence of suitable restriction sites.

1.2.b. Deletion fragments

A series of deletion subclones of the complementing *SalI-HinDIII* fragment were created to determine the minimum fragment required for the complementation of AM77. Deletions from the *HinDIII* site were made by *Bal31* nuclease digestion. DNA of plasmid pAD300 was prepared by the LiCl method and digested with *HinDIII*. Aliquots of the DNA were then digested by *Bal31* for different time periods to give a variety of fragment lengths. After end repair the *Bal31* digested DNA was cut by *SalI* and then ligated into *SalI-SmaI* cut pUC19. Prior to transformation into DH5α the ligated DNA was digested with *NruI* to remove any remaining pBR322. The transformants were selected on Amp + XG + IPTG plates. DNA was prepared from white colonies and digested with *EcoRI*. Putative *Bal31* deleted subclones had a constant 4.04kb fragment and a variable length fragment. From the 110 colonies that were screened, five, representing a range of *Bal31* deletion fragment sizes, were selected. To act as a positive control for the *Bal31* deletion subclones, and to enable further subcloning, the *SalI-HinDIII* fragment from pAD300 was subcloned into *SalI-HinDIII* cut pUC19, resulting in pMM300. pMM450 was the product of *EcoRI* digestion of pMM300 followed by self ligation. All constructs are shown in figure 3.2A.

AM77 was transformed with pMM300 and all deletion plasmids referred to above. Plasmids were scored for complementation of AM77's mutant phenotype in both the

presence and absence of added NaCl. The results are shown in figure 3.2B. The only plasmids that were unable to complement growth were pMM305 and pMM450, both of which contained deletions of part of *cysQ*. In all cases deletion plasmids that restored growth on M63 alone also restored growth in the presence of 0.35M NaCl.

1.3. Mapping of mini Tn*10* insertions

The transposon insertion of AM77, *otsC::*Tn*10kan* had been cloned by McLellan into plasmid pAD77 (see figure 5.2) and the insertion site was mapped by him to 200bp below *cysQ* (*amtA*). The insertion site of *otsC::*Tn*10kan* was remapped in this study by *PstI-BamHI* double digestion which yielded a restriction fragment of approximately 160bp. The size of this fragment showed *otsC::*Tn*10kan* to coincide with the end of *cysQ* (figure 3.1A), although it could not be determined whether the insertion was in, or immediately after, *cysQ* .

1.4. Complementation by amino acid supplementation

Both AM77 and mutations causing loss of complementation by pAD300 had been mapped by McClellan to the region immediately downstream of a gene, *cysQ*, which although originally thought to be involved in ammonium uptake, was later found to be necessary for cysteine biosynthesis. On minimal media *CysQ* mutations give a "leaky" auxotrophic phenotype of slow growth, which is relieved by addition of cysteine. Because of the proximity of *otsC::*Tn*10kan* to *cysQ*, and the growth retardation observed on minimal media even in the absence of osmotic stress, the effect of adding cysteine was examined. Addition of cysteine to 0.3mM was found to restore approximately wild type growth to AM77 and remove its osmosensitivity in all conditions tested. Methionine also restored growth, but only in the absence of osmotic stress. However, no other amino acids were able to complement the mutant phenotype of AM77. Other forms of reduced sulphur were also found to suppress AM77's mutant phenotype, particularly potassium thiosulphate. Sodium sulphite, which had been reported to fully complement *cysQ* mutants by Neuwald and coworkers (1991), complemented AM77, but poorly. All results are summarised in figure 3.4 and photographs of growth are shown in figure 3.6.

Reduced sulphur sources were also added to the parent strain W3110, results for which are shown in figure 3.9. Both potassium thiosulphate and cysteine were observed to enhance the growth of W3110 in the presence of added NaCl.

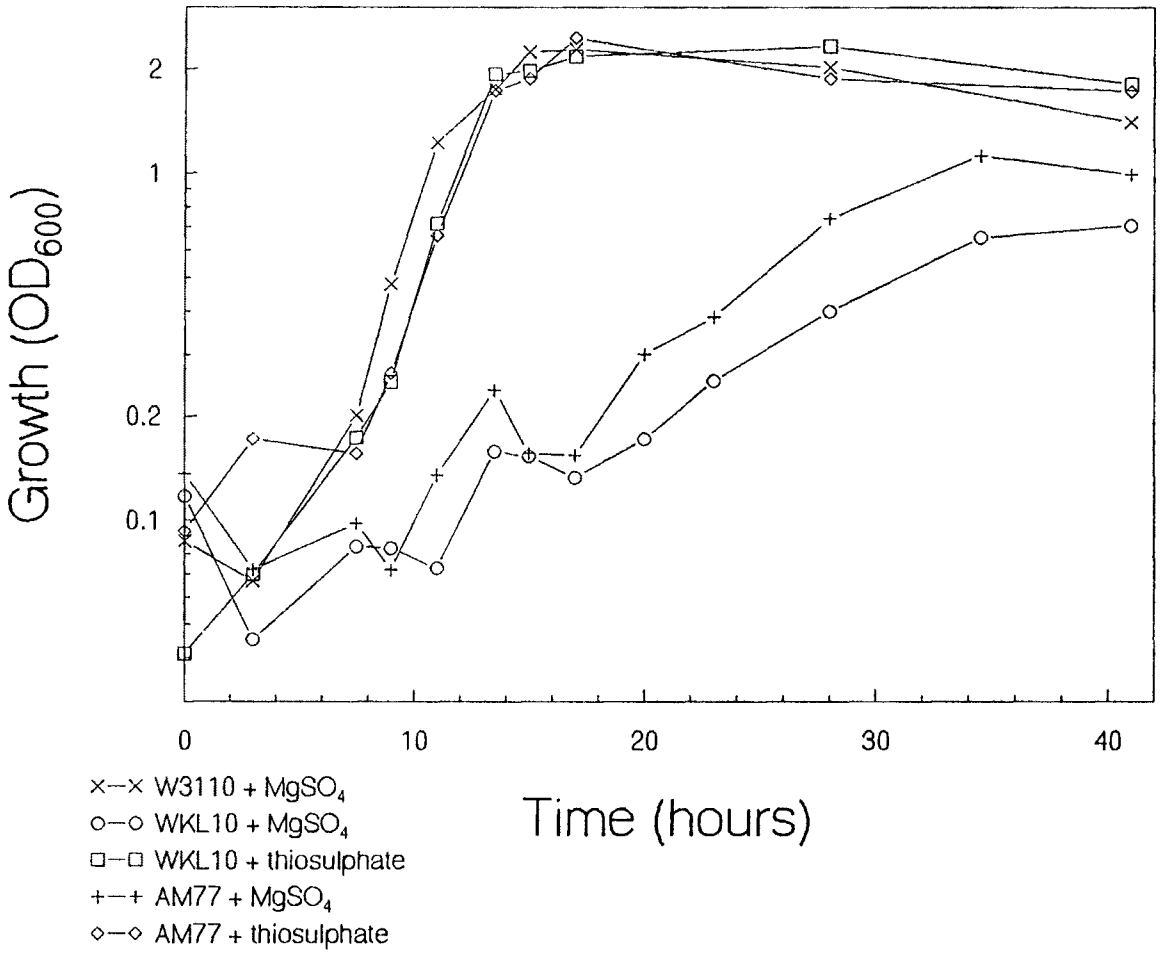


Figure 3.3. Phenotype of AM77, WKL10 and W3110 in liquid culture, and complementation by thiosulphate. Overnight cultures were washed twice in 1 x M9 salts and then M9 cultures were inoculated at 1/100. All readings are the average of three independent cultures.

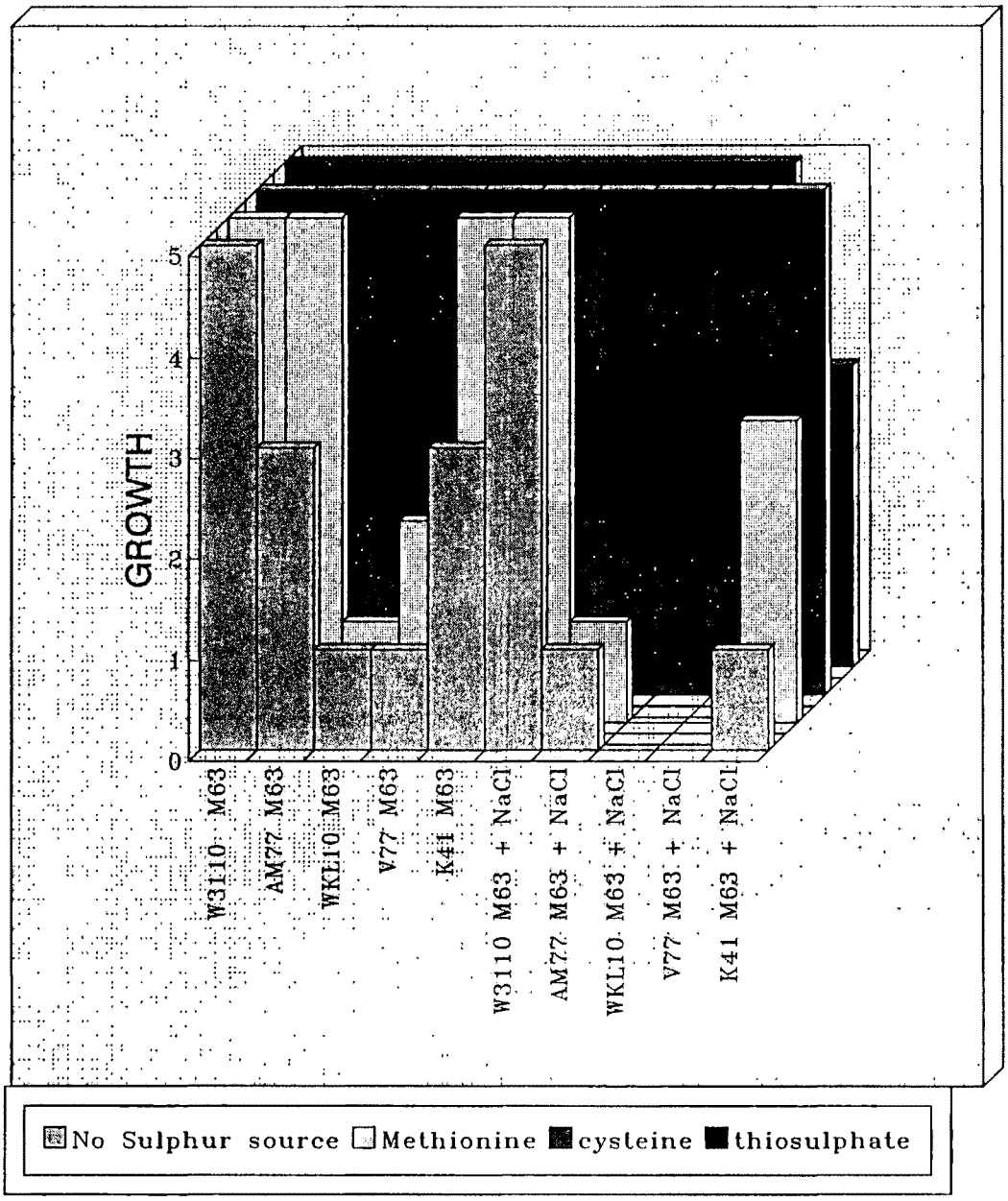


Figure 3.4. Phenotype of W3110, AM77, WKL10, V77 and K41 with different reduced sulphur sources. These results are for M63 plates in both the presence and absence of added NaCl. Growth was scored on a subjective scale:

5	wild type
4	slight growth retardation
3	clear growth retardation
2	faint growth, very small single colonies visible
1	very faint growth, no single colonies
0	no growth

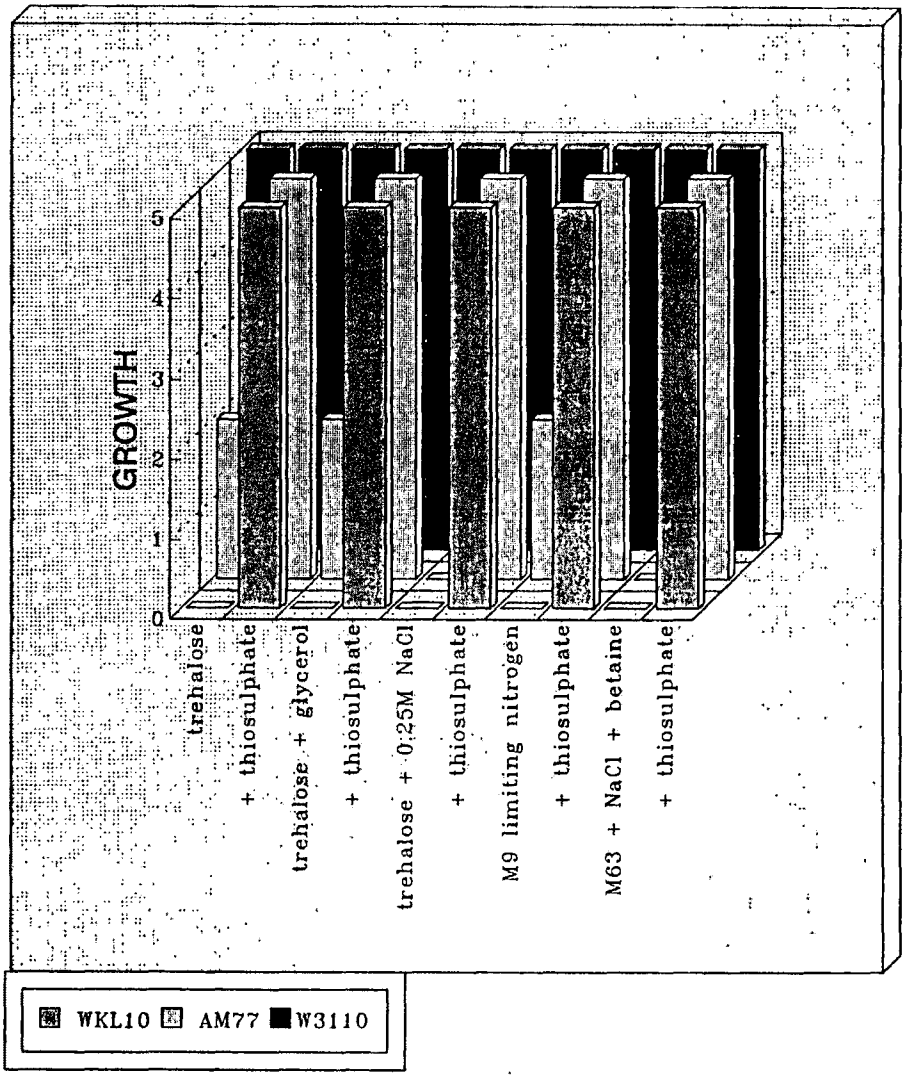


Figure 3.5. Phenotype of W3110, AM77 and WKL10 with trehalose or limiting nitrogen media. Bacteria were grown in the following conditions in either the presence or absence of thiosulphate:

M63 with trehalose as the sole carbon source

M63 with trehalose + glycerol as the carbon sources

M63 with trehalose as the carbon source + 0.25M NaCl

M9 with limiting nitrogen

M63 + 0.35M NaCl + glycine betaine

Growth was scored on the following subjective scale:

5	wild type
4	slight growth retardation
3	clear growth retardation
2	faint growth, very small single colonies visible
1	very faint growth, no single colonies
o	no growth

Figure 3.6 Growth of mutants AM77 and V77 on M63 media and M63 + 0.35M NaCl, in the presence and absence of complementing plasmid pAD300, and of potassium thiosulphate as a reduced sulphur source.

1. W3110 (wild type)
2. WKL10 (*cysQ*$_{10}$::Tn*10-LK*)
3. V77 pAD300
4. V77
5. AM77 pAD300
6. AM77

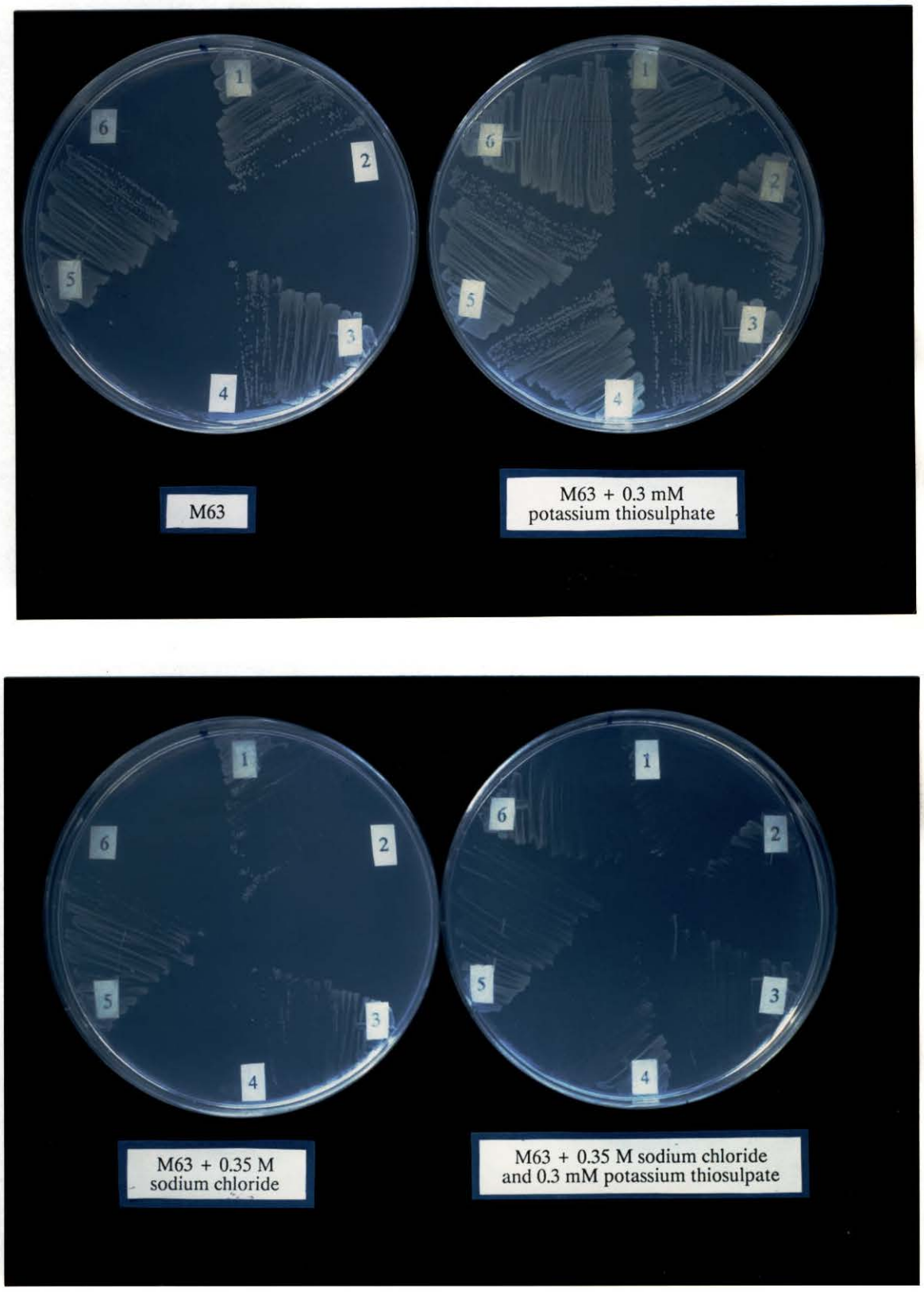


Figure 3.6. Growth of W3110, WKL10, AM77 and V77.

Figure 3.7. Growth of strains containing introduced chromosomal mini Tn*10* insertions, on M63 or M63 + 0.35 M NaCl, and in the presence and absence of potassium thiosulphate as a sulphur source.

1. W3110 (wild type)
2. MM6
3. WCH7
4. WKL8
5. WKL10
6. AM77

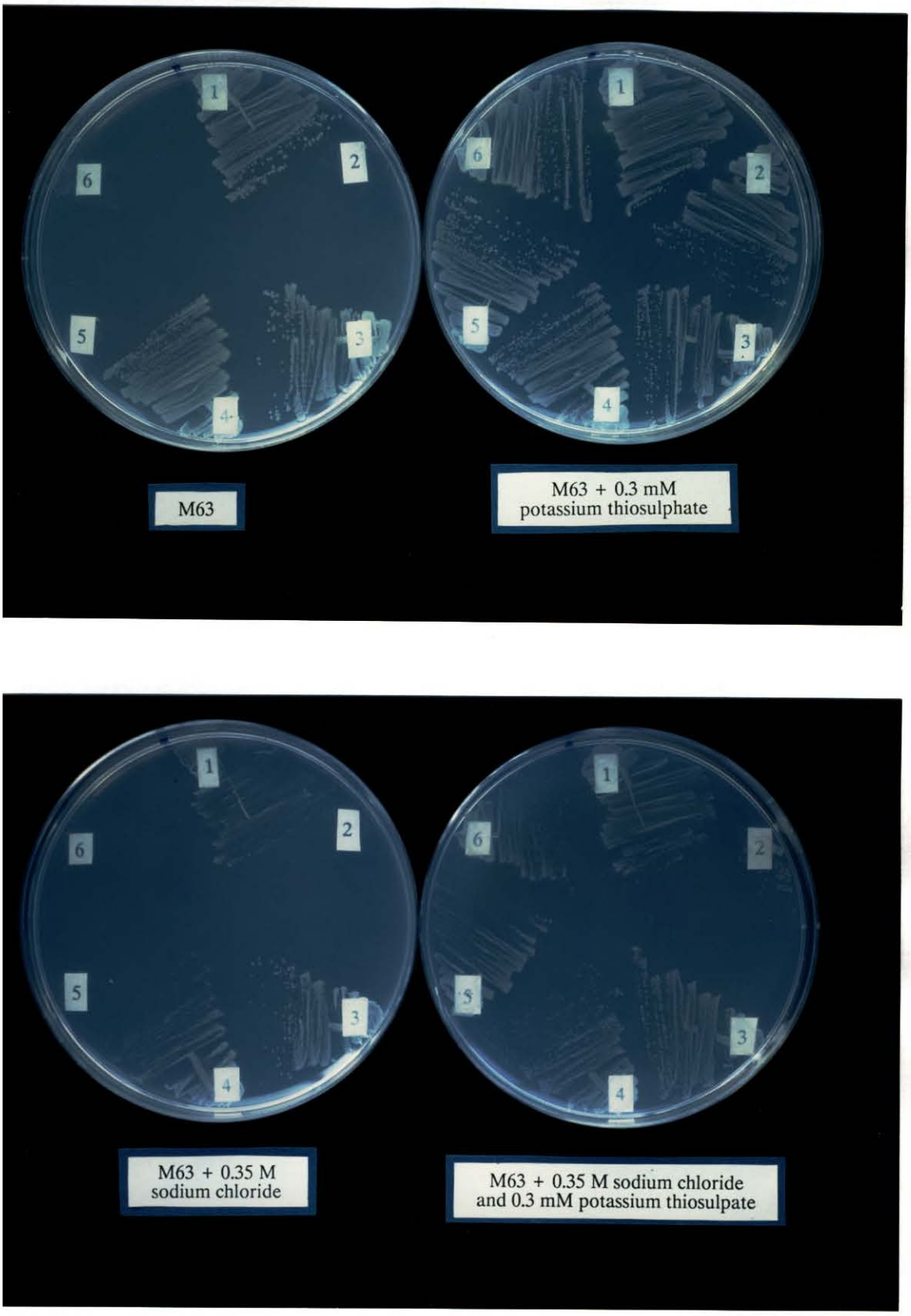


Figure 3.7. Growth of chromosomal insertions.

Figure 3.8. Growth of K41 and its derivative MM17 (containing K41 rescued Kan^r marker) on minimal media with or without Potassiu thiosulphate, and/or complementing plasmid pAD300.

1. W3110 (wild type)
2. WKL10 (*cysQ$_{10}$*::Tn*10-LK*)
3. MM17 pAD300
4. MM17
5. K41 pAD300
6. K41

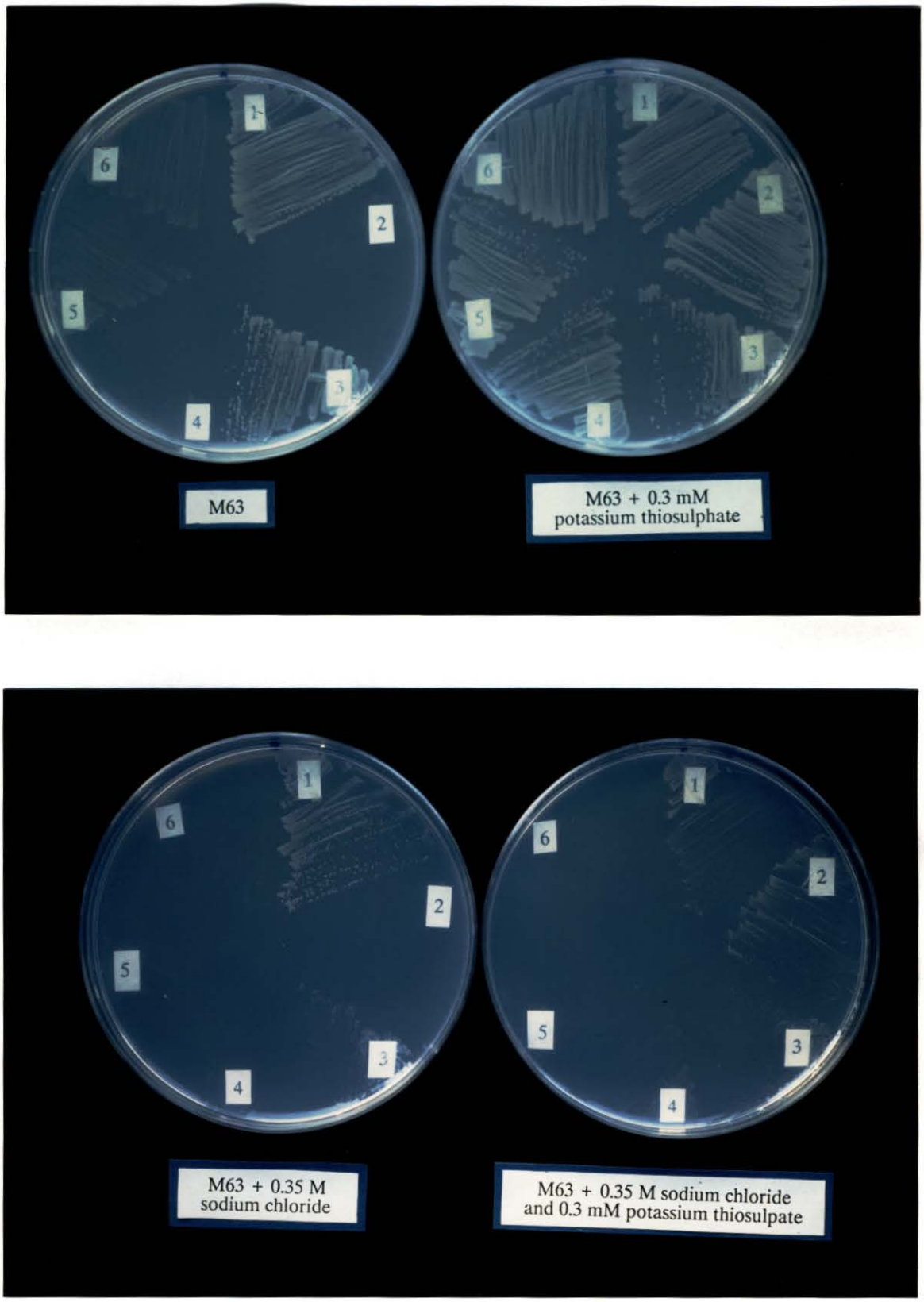


Figure 3.8. Growth of K41 and derivatives.

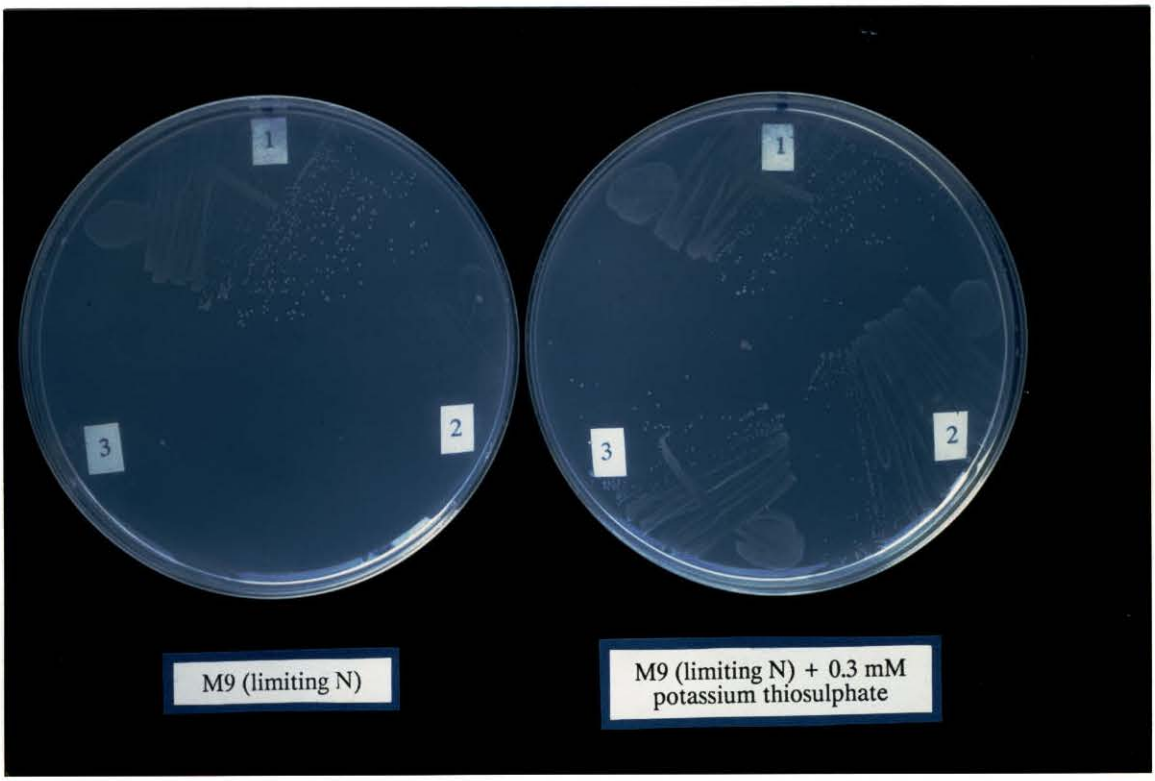


Figure 3.9. Growth of W3110, WKL10 and AM77 on M9 media with limiting nitrogen in the presence and absence of potassium thiosulphate as a reduced sulphur source. The numbers on the plate are:

1. W3110
2. AM77
3. WKL10

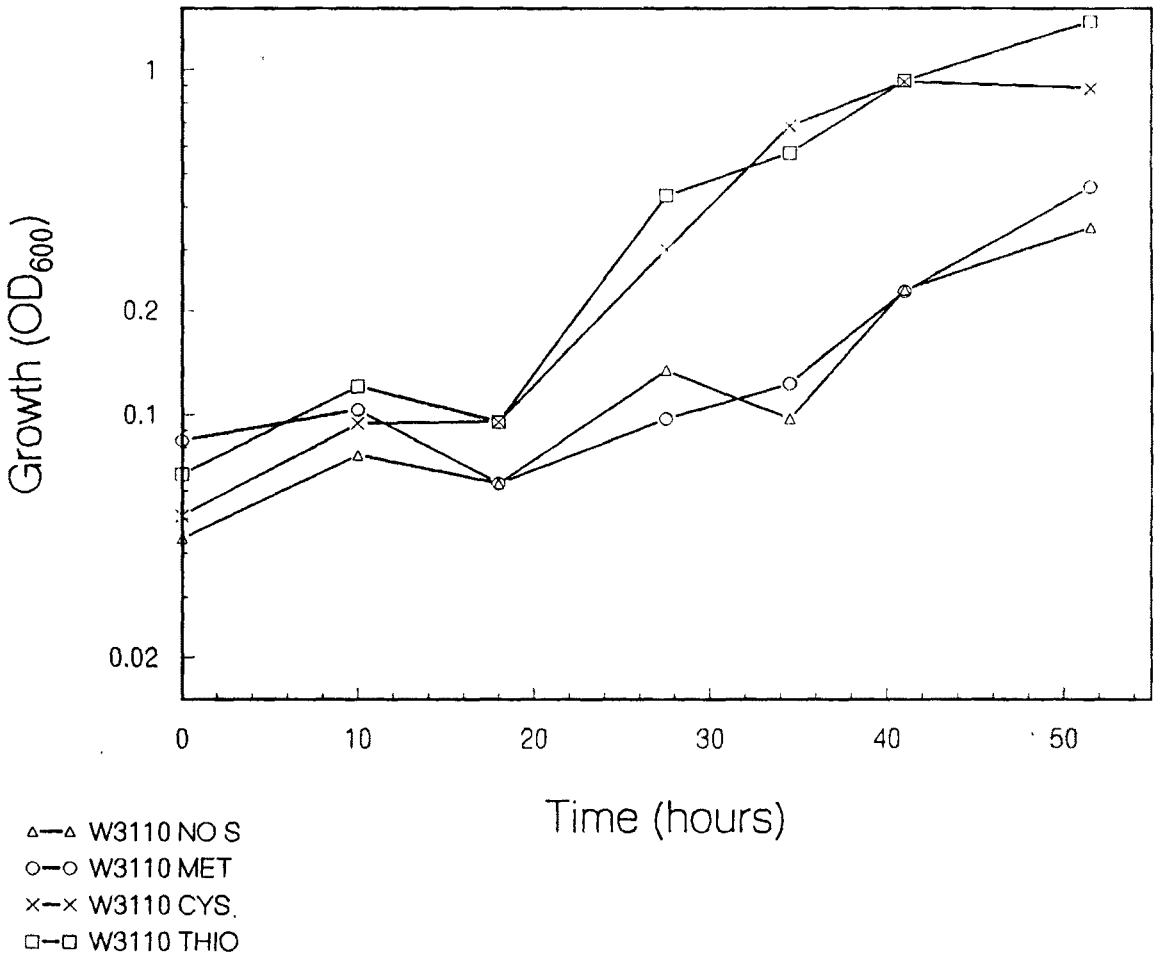


Figure 3.10. The effect of reduced sulphur sources on the growth of W3110 in high osmolarity liquid cultures. Washed resuspended overnight cultures were inoculated into M9 media, with either no added sulphur source, or with methionine, cysteine or thiosulphate supplied. These results are the averages of triplicate cultures.

2. CHROMOSOMAL INSERTIONS

The results from complementation and restriction mapping suggested that AM77 was a *cysQ* mutant. However, mutations in pAD300 up to 1.2kb below the 3' end of *cysQ* prevented complementation in conditions of osmotic stress. To clarify both the role of the downstream region and the genotype of AM77 it was decided to transduce three mini Tn*10* insertions from pAD300 into the chromosome to obtain single copy chromosomal insertions. The three insertions chosen *cysQ*$_{8}$::Tn*10-LK*, *cysQ*$_{10}$::Tn*10-LK* and *CH7*::Tn*10cam*, were from plasmids pKL8, pKL10 and pCH7 respectively. All failed to complement AM77 for growth on M63 + 0.35M NaCl. *cysQ*$_{8}$::Tn*10-LK* and *cysQ*$_{10}$::Tn*10-LK* were also active *lacZ* translational fusions which differed in β-galactosidase activity (see chapter 1 section 3.5), suggesting that they were inserted in different loci. It was thought that obtaining chromosomal insertions of these fusions would facilitate study of the regulation of the gene(s) into which they were inserted.

2.1. Strategy for obtaining insertions

The strategy used was that of Kulakauskas *et al.* (1991) who obtained single copy chromosomal insertions by using the λ phage library of Yuji Kohara (Kohara *et al.* 1991). A λ lysate of the Kohara phage homologous to the region of interest was grown on a permissive host containing plasmids bearing the mutation to be transduced. Homologous recombination into the phage occured and the phage was then used to transduce the mutation and a selectible marker to a recipient strain. A schematic of this strategy is shown in figure 3.11. This strategy can also be used for transduction of chromosomal mutations, much as for P1 transduction, except that only mutations bounded by DNA homologous to the insert in the phage will be transduced.

The Kohara phage selected for use in transduction was λ K656, from which pAD300 was originally cloned. K656 lysates were prepared on 71-18 containing pKL8, pKL10 or pCH7. As controls λ K446 lysates of pKL10, pCH7 and AM77 were also prepared (K446 is another Kohara phage containing an insert of DNA from 58.4 minutes, which has no homology to that of K656). Titres for all phages were 10^{10}-10^{11} pfu /ml. The initial recipient strains for transduction were ER1647 (*recD*) or ZK592 (*recBC sbcB*). Both *recD* and *recBC sbcB* strains destabilise colE1 based plasmids resulting in an increased frequency of chromosomal insertions (Kulakauskas *et al.* 1991).

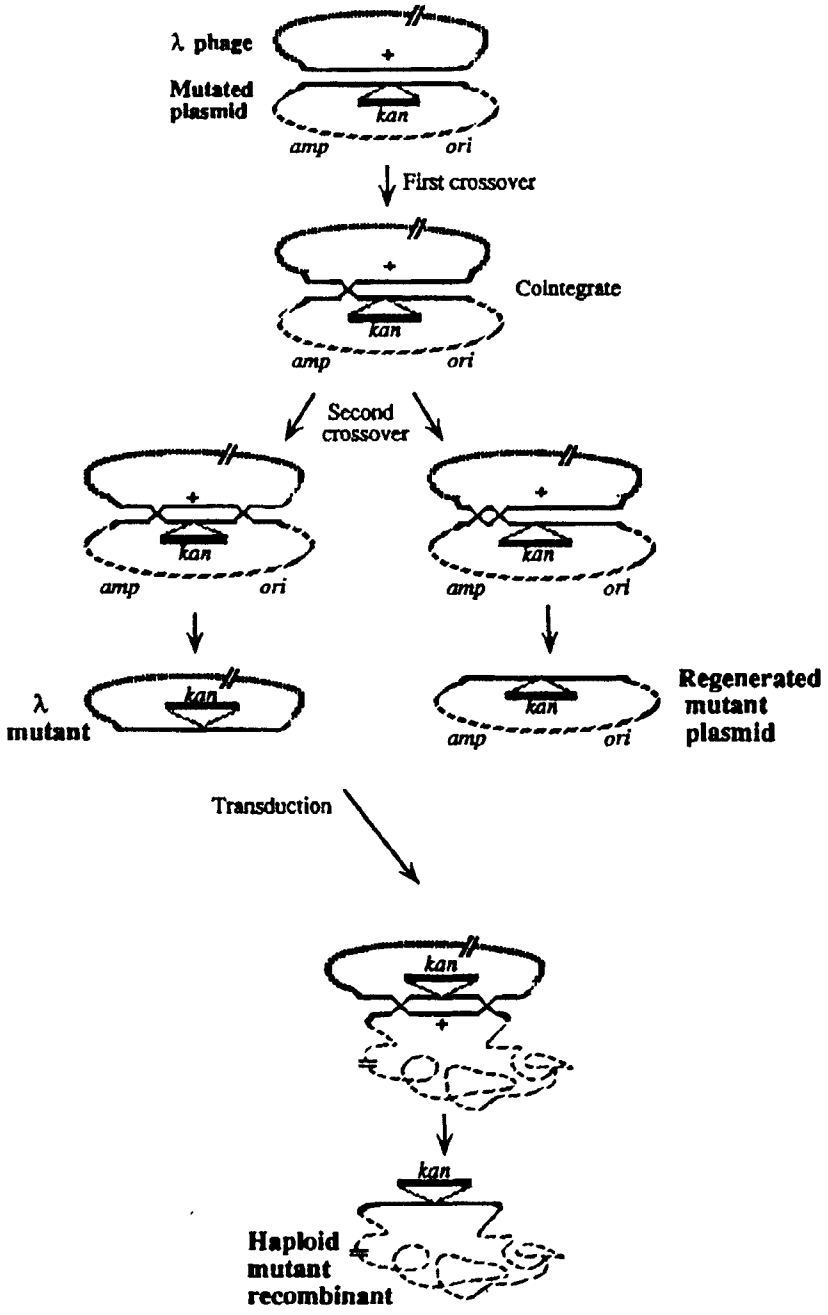


Fig 3.11. λ- mediated transduction of plasmid bourne alleles. Symbols: ▬, bacterial sequences cloned in plasmid or λ phage, or corresponding chromosomal segment; ▬ *kan* insert seqences; ···· plasmid vector; ∿ bacterial chromosome; ▬ λ phage vector. From Kulakauskas *et. al.* (1991).

2.2. Transduction into the chromosome

The resulting strains from all transductions are listed in table 3.1, while a map showing the origin of the insertions is given in figure 3.1A.

2.2.a. *cysQ8::*Tn*10-LK*

The insertion *cysQ8::*Tn*10-LK* was transduced into ER1647 with selection for the Kan^r of the lac-kan fusion. Five of fourteen Kan^r colonies were tested for $\lambda^s$and Amp^s to select against the presence of lysogenic λ, or the plasmid, respectively. Plasmid DNA preparations confirmed the absence of pKL8 DNA. One of the five, designated MM1, was found to be λ^s and Amp^s and did not contain pKL8. It was concluded that MM1 contained a single copy chromosomal insertion of *cysQ8::*Tn*10-LK*.

2.2.b. *cysQ10::*Tn*10-LK*

Transduction of *cysQ10::*Tn*10-LK* into ZK592 by K656 lysate yielded 42 Kan^r transductants of which 36 were λ^s. Five of the λ^s transductants were selected, all of which were found to be Amp^s. One of these Amp^s λ^s transductants was designated MM2. No Kan^r colonies were obtained from transductions using K446 lysates.

Table 3.1. Summary of transductions of mini Tn*10* insertions from plasmids into the chromosome.

strain	insertion	donor	strain background	λ^s	Amp^s
MM1	*cysQ8*::Tn*10-LK*	71-18 pKL8	ER1647	√	√
71KL8	*cysQ8*::Tn*10-LK*	MM1	71-18	√	√
WKL8	*cysQ8*::Tn*10-LK*	MM1	W3110	√	√
MM2	*cysQ10*::Tn*10-LK*	71-18 pKL10	ZK592	√	√
71KL10	*cysQ10*::Tn*10-LK*	MM2	71-18	√	√
WKL10	*cysQ10*::Tn*10-LK*	MM2	W3110	√	√
MM3	*CH7*::Tn*10Cam*	71-18 pCH7	ZK592	√	√
71CH7	*CH7*::Tn*10Cam*	MM3	71-18	√	√
WCH7	*CH7*::Tn*10Cam*	MM3	W3110	√	√

2.2.c. *CH7::*Tn*10cam*

A single copy chromosomal insertion of *CH7::*Tn*10cam* proved difficult to obtain, with five transductions being attempted before one was recovered. In all five transductions there were large numbers of Cam^r colonies (100-500 /plate). These colonies were heterogeneous in size and they were all λ^r and Amp^r. None were obtained from non transformed recipients or using K446 lysate, suggesting they were due to a K656 specific transduction event. On the fifth transduction five larger colonies were obtained which it was concluded were chromosomal insertions as they were λ^S and Amp^S; one of these was designated MM3.

2.3. Transduction to W3110 and 71-18

K656 lysates of MM1, MM2 and MM3 were prepared. As a control a K446 lysate of MM3 was also prepared. Transduction of W3110 and 71-18 gave transductants for all K656 lysates, but not for K446. All transductants of MM1 and MM2 were Kan^r, Amp^S and λ^S, while those of MM3 were Cam^r, λ^S and Amp^S. The resulting strains from transduction of the *cysQ*$_8$*::*Tn*10-LK*, *cysQ*$_{10}$*::*Tn*10-LK* and *CH7::*Tn*10cam* insertions into W3110 were WKL8, WKL10 and WCH7 respectively, while transduction into 71-18 yielded 71KL8, 71KL10 and 71CH7.

2.4. PCR mapping of insertions

PCR mapping was used to confirm that the transduced antibiotic resistances were due to chromosomal insertions of mini Tn*10*, and that the mini Tn*10* insertions were in the expected positions relative to *cysQ*. The methodology used is fully described in section 5.2. PCR of single colonies of WKL10, WKL8, 71KL10, 71KL8 and 71CH7 gave the expected PCR fragments (see figure 3.18A). No PCR fragments were obtained from the parent strains 71-18 or W3110. PCR Mapping of the *cysQ*$_8$*::*Tn*10-LK* insertion showed it to be closer to *cysQ* than was previously thought. The PCR products of AM77 and 71KL8 were indistinguishable in length even when digested with *PstI* to allow fine mapping.

2.5. Phenotype of chromosomal insertions

Photographs of the typical growth of the chromosomal insertions referred to in this section are shown in figure 3.7.

2.5.a. WKL10

WKL10 failed to grow on M63 + 0.35M NaCl plates and growth on M63 plates was very faint even after three days. WKL10 accumulated suppressor mutations, although not as frequently as AM77. Addition of cysteine or potassium thiosulphate, but not methionine, restored growth to wild type levels in both the presence and absence of added NaCl. WKL10 was complemented by the plasmids pAD300, pMM300 and pMM410, but not by pMM305 or 450. These results are summarised in figure 3.2B.

The phenotype of WKL10 was also examined in M9 liquid media, with these results being shown in figure 3.3. Both doubling time and growth lag were increased in M9 alone. Thiosulphate restored growth to wild type levels while methionine and cysteine only partially complemented. Addition of 0.35M NaCl completely halted growth. Phenotypically 71KL10 grew like WKL10.

2.5.b. WKL8

Phenotypically WKL8 grew like wild type. Growth of WKL8 was indistinguishable from W3110 in all conditions tested which included solid and liquid minimal media in both the presence and absence of added NaCl. Growth of 71KL8 was qualitatively identical to that of WKL10.

2.5.c. WCH7

WCH7 and 71CH7 also appeared to behave as wild type in the conditions tested. Growth of WCH7 was examined on all media listed in figures 3.4 and 3.5, but no differences in growth compared with W3110 were observed.

2.6. *CH7::*Tn*10cam* insertion in combination with other insertions

As *CH7::*Tn*10cam* had originally been selected as an insertion that prevented complementation of AM77 by pAD300 in conditions of osmotic stress, it was thought necessary to determine whether a combination of *CH7::*Tn*10cam* with the transposon insertion from AM77, *otsC::*Tn*10kan*, would result in a change in phenotype. To do this a P1*kc* lysate of AM77 was used to transduce WCH7 to Kan^r. Several hundred Cam^r, Kan^r colonies were obtained, of which four were tested for auxotrophy. All four, one of which was designated MM6, had retarded growth on M63 plates and were complemented by addition of potassium thiosulphate, confirming transduction of *otsC::*Tn*10kan* had occurred. MM6 was phenotypically indistinguishable from AM77 on all media listed in figure 3.4. MM6 was also

observed to give rise to suppressor mutations when grown in liquid minimal media, as for AM77.

The *CH7::*Tn*10cam* insertion was transduced into 71KL8 and 71KL10 resulting in strains MM18 and MM19 respectively. Both MM18 and MM19 behaviour in minimal conditions were identical to their respective parent strains, 71KL8 and 71KL10.

3. SEQUENCING

The junction points of the transposon insertions *otsC::*Tn*10kan* (AM77), *cysQ$_8$::*Tn*10-LK* (pKL8) and *cysQ$_{10}$::*Tn*10-LK* (pKL10) were sequenced. The strategy used was to isolate a single transposon end by subcloning, followed by double stranded dideoxy sequencing using the outward facing Tn*10*-IS10R primer previously synthesised for PCR (section 5.2). This strategy was used as the inverted repeats of mini Tn*10* prevent double stranded sequencing of insertion points (Kleckner *et al.* 1991), therefore it was necessary to isolate a single transposon end. The insertion junctions of *otsC::*Tn*10kan* and *cysQ$_8$::*Tn*10-LK* were sequenced twice from seperately isolated DNA to confirm no cross contamination had occurred.

3.1. Subcloning

DNA from pAD77 was digested with *HinDIII* and electrophoresed. The small *HinDIII* fragment was excised and the DNA eluted and ligated into *HinDIII* cut alkaline phosphatase treated pUC18. DNA of five Amp^r transformants was digested with *EcoRI*. All contained the expected fragment, four in one orientation and one in the other. One of these colonies was designated pMM77.

The insertions *cysQ$_8$::*Tn*10-lacZ* and *cysQ$_{10}$::*Tn*10-lacZ* were subcloned from pKL8 and pKL10 respectively. The *SalI-HinDIII* fragments containing *cysQ* and the complete *lacZ* fusion were excised from an agarose gel, eluted, and then ligated into *SalI-HinDIII* cut pUC18. Transformants giving the expected restriction fragments and having *lacZ* activity were designated pMM80 and pMM100 (from pKL8 and pKL10 respectively). Subcloning of pMM80 and pMM100 is shown in appendix V figure 5.4.

3.2. Insertion Positions

The sequences at the junction points of *otsC::*Tn*10kan*, *cysQ$_8$::*Tn*10-LK* and *cysQ$_{10}$::*Tn*10-LK* are shown in figure 3.12. All three insertions were into *cysQ*, which had previously been sequenced (Jayakumar *et al.* 1991, Neuwald *et al.* 1992). Both *otsC::*Tn*10kan*, and *cysQ$_8$::*Tn*10-LK*, had mini Tn*10* insertions 9 bp from the 3' end of *cysQ*, while the insertion of *cysQ$_{10}$::*Tn*10-LK* was exactly 120 bp from the 3' end. Consequently the genotypes of these three insertions are referred to as *cysQ$_{77}$::*Tn*10kan* , *cysQ$_8$::*Tn*10-LK* and *cysQ$_{10}$::*Tn*10-LK* respectively. The insertion positions on the map of *cysQ* are shown in figure 3.1A.

Figure 3.12. Sequencing of transposon insertion junction points of pMM77, pMM80 and pMM100. The underlined sequence is the chromosomal sequence up to the transposon junction point.

a. Transposon insertion junction in pMM77

sequence read	3'-AGGCCCCAAGTCTCACGACTACTTAGGGG-5'
IS10 sequence	3'-GACTACTTAGGGG-5'
cysQ sequence (- strand)	3'-AGGCCCCAAGTCTCACAGATAAATGATTTA-5'
cysQ sequence (+ strand)	5'-TCCGGGGTTCAGAGTGTCTATTTACTAAAT-3'

b. Transposon insertion junction in pMM80

sequence read	3'-AGGCCCCAAGTCTCACGACTACTTAGGGG-5'
IS10 sequence	3'-GACTACTTAGGGG-3'
cysQ sequence (- strand)	3'-AGGCCCCAAGTCTCACAGATAAATGATTTA-5'
cysQ sequence (+ strand)	5'-TCCGGGGTTCAGAGTGTCTATTTACTAAAT-3'

c. Transposon insertion junction in pMM100

sequence read	3'-AAACCCTGTGGCGGGACTACTT-5'
IS10 sequence	GACTACTT-3'
cysQ sequence (- strand)	3'-AAACCCTGTGGCGGCACCTGTA-5'
cysQ sequence (+ strand)	5'-TTTGGGACACCGCCGTGGACAT-3'

4. β-GALACTOSIDASE ACTIVITY OF THE *CYSQ-LACZ* FUSION STRAIN, 71KL8

Translational *lacZ* fusions *cysQ8*::Tn*10-LK* and *cysQ10*::Tn*10-LK* were both shown by sequencing to be insertions into *cysQ*. Both fusions had been isolated in single copy chromosomal insertions, in strains 71KL8 and 71KL10 respectively (described in section 2). 71KL8 and 71KL10 were used to examine the transcriptional and translational regulation of *cysQ*, about which nothing has been published. Although both insertions were already available on plasmids, it was thought that single copy insertions would more accurately reflect the normal cellular conditions, without the influence of increased gene dosage.

Initial observations of the β-galactosidase activity of strains 71KL8 and 71KL10 showed both fusions to be active in both rich and minimal conditions, and in both liquid cultures and on plates, whereas the parent strain, 71-18, had no β-galactosidase activity. It was observed that 71KL8 had greater activity than 71KL10, confirming the observations of McLellan (1992), who noted the same relationship for pKL8 and pKL10. Quantitative analysis in liquid cultures confirmed that the β-galactosidase activity of 71KL10 was appreciably less than that of 71KL8; in all conditions tested activity of 71KL10 was approximately one quarter that of 71KL8. A comparison of the time course of β-galactosidase induction for either strain in rich media is shown in figure 3.13. As both fusions were insertions into *cysQ* and displayed similar induction characteristics, and because of its uniformly higher β-galactosidase activity, 71KL8 (fusion *cysQ8*::Tn*10-LK*) was selected for all further assays of *cysQ-lacZ* fusion activity. 71KL8 was also shown to have wild type, or very close to wild type, growth, which gave the advantage of being able to assay β-galactosidase activity in media that would not support the growth of 71KL10.

Initial attempts to determine factors affecting 71KL8 β-galactosidase activity during exponential growth were largely unsuccessful with large, seemingly random, fluctuations in activity being observed. The reason for this was discovered when the time course of the β-galactosidase induction in 71KL8 and 71KL10 was determined. 71KL8 expression was found to be at its highest during stationary phase, with low level expression during exponential growth in rich media (LB), followed by 15-20 x induction after the onset of stationary phase. The same relationship was also observed for 71KL10, albeit with lower β-galactosidase levels. As the levels of the CysQ-β-galactosidase fusion protein were so high during stationary phase, this

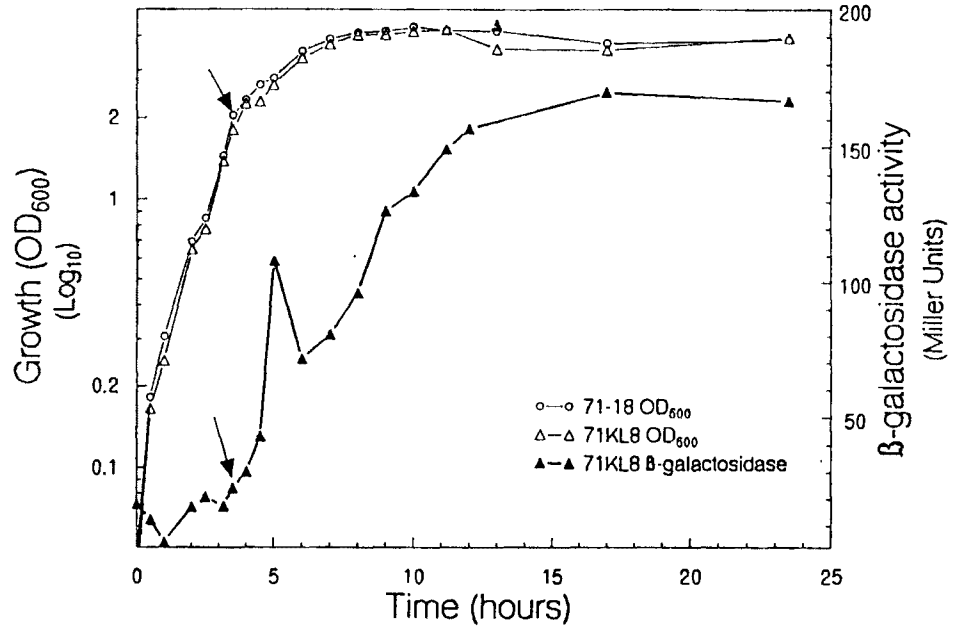
suggested that the fluctuations observed in β-galactosidase levels during early to mid exponential growth were caused by high levels of fusion protein still being present from the previous stationary phase. For this reason subsequent growth phase β-galactosidase activity determinations were done using cells which had been purified by at least ten generations of exponential growth prior to inoculation into the media to be assayed. Generally both exponential and stationary phase expression levels were determined.

β-galactosidase levels were also observed to stay high, even after several days of growth in stationary phase. In figure 3.16 β-galactosidase activity is plotted against viable cell count over a four day period. β-galactosidase activity was observed to stay approximately equal over this time period, whereas viable cell count decreased by 90%.

4.1. Activity in minimal conditions

The β-galactosidase activity of 71KL8 was approximately four times higher during growth in minimal media than it was in rich media. It was also noted that, as occurred in rich media, there was induction of activity upon entry into stationary phase. In minimal conditions the degree of induction was less, about 12 fold, however the maximum β-galactosidase activities were greater than in rich media. The effect of addition of cysteine, methionine and thiosulphate were also assessed, as summarised in figure 3.14, as are the results for the remainder of section 4.1. All assays are the average of at least two independent liquid cultures, with all β-galactosidase assays performed in duplicate, and results being expressed in Miller units. β-galactosidase activity was decreased by all three sulphur sources, but only during exponential growth; stationary phase activities were unchanged. Thiosulphate caused the greatest decrease, to 11.3 Miller units, only slightly greater than that measured during growth in rich media. Methionine caused an intermediate decrease in activity, while cysteine had a relatively minor effect. The effect of trehalose as a carbon source was also assessed, with a small difference in β-galactosidase activity being observed during exponential growth. Activity was also decreased during stationary phase. Stationary phase expression levels in M63 + 0.35M NaCl were also assessed. Forty-eight hours after inoculation an activity of 313 Miller units was observed.

a. Growth and ß-galactosidase activity in rich media.



Arrows mark the exit from exponential growth

b. Growth and ß-galactosidase activity in minimal media

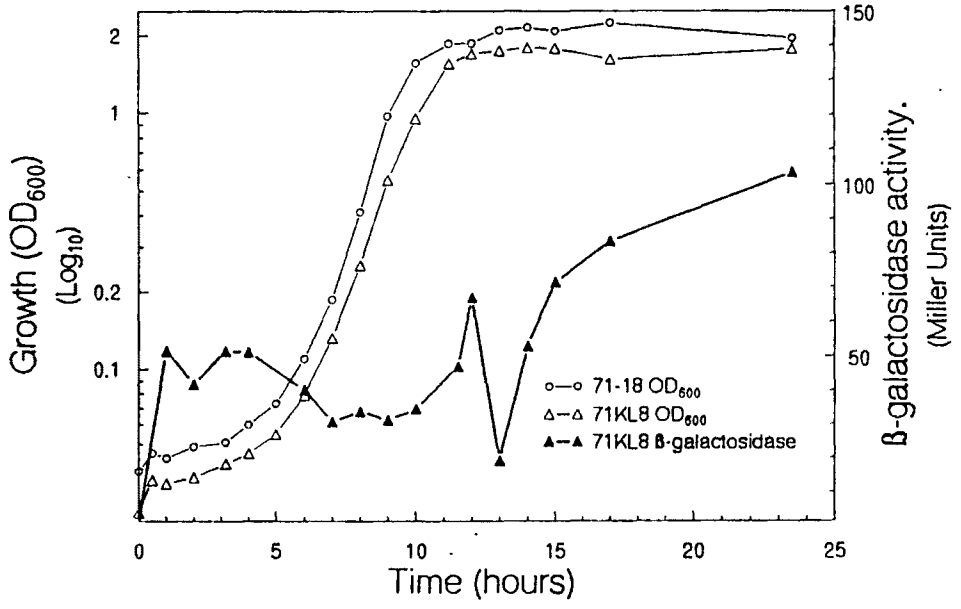


Figure 3.13. Time course of β-galactosidase induction. 71KL8 was purified for 10 generations in exponential growth and then inoculated into either M63 (after washing) or LB media. The results presented are the average of two independent cultures, and each β-galactosidase assay was done in duplicate.

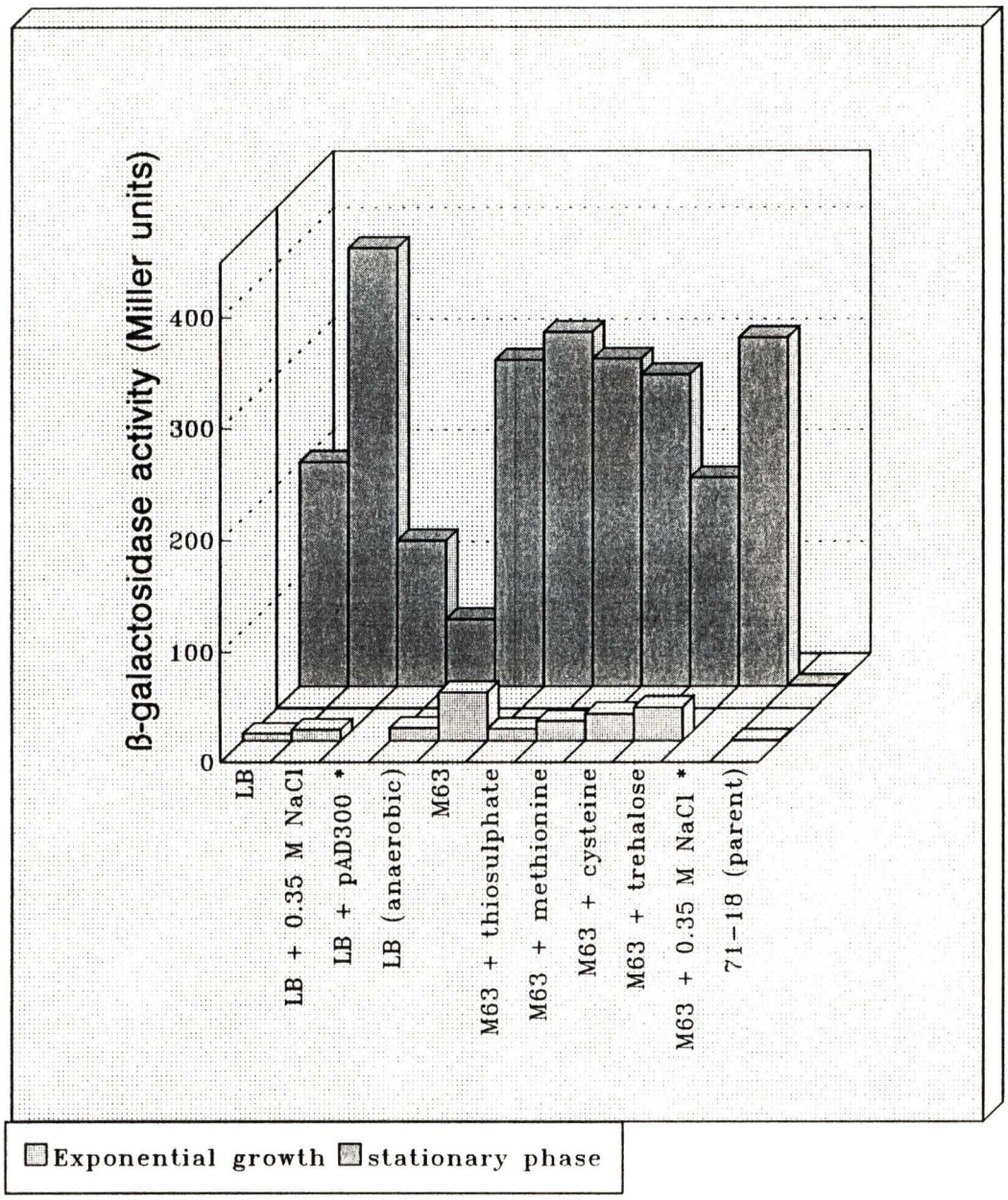


Figure 3.14. Effect of different media on β–galactosidase induction of 71KL8. The β-galactosidase activity of 71KL8 was determined during exponential growth (except for those marked with *) and in early-mid stationary phase. All results are from duplicate cultures and β-galactosidase activity of each culture was determined in duplicate. Exponential cultures were purified in exponential growth for at least 10 generations prior to inoculation.

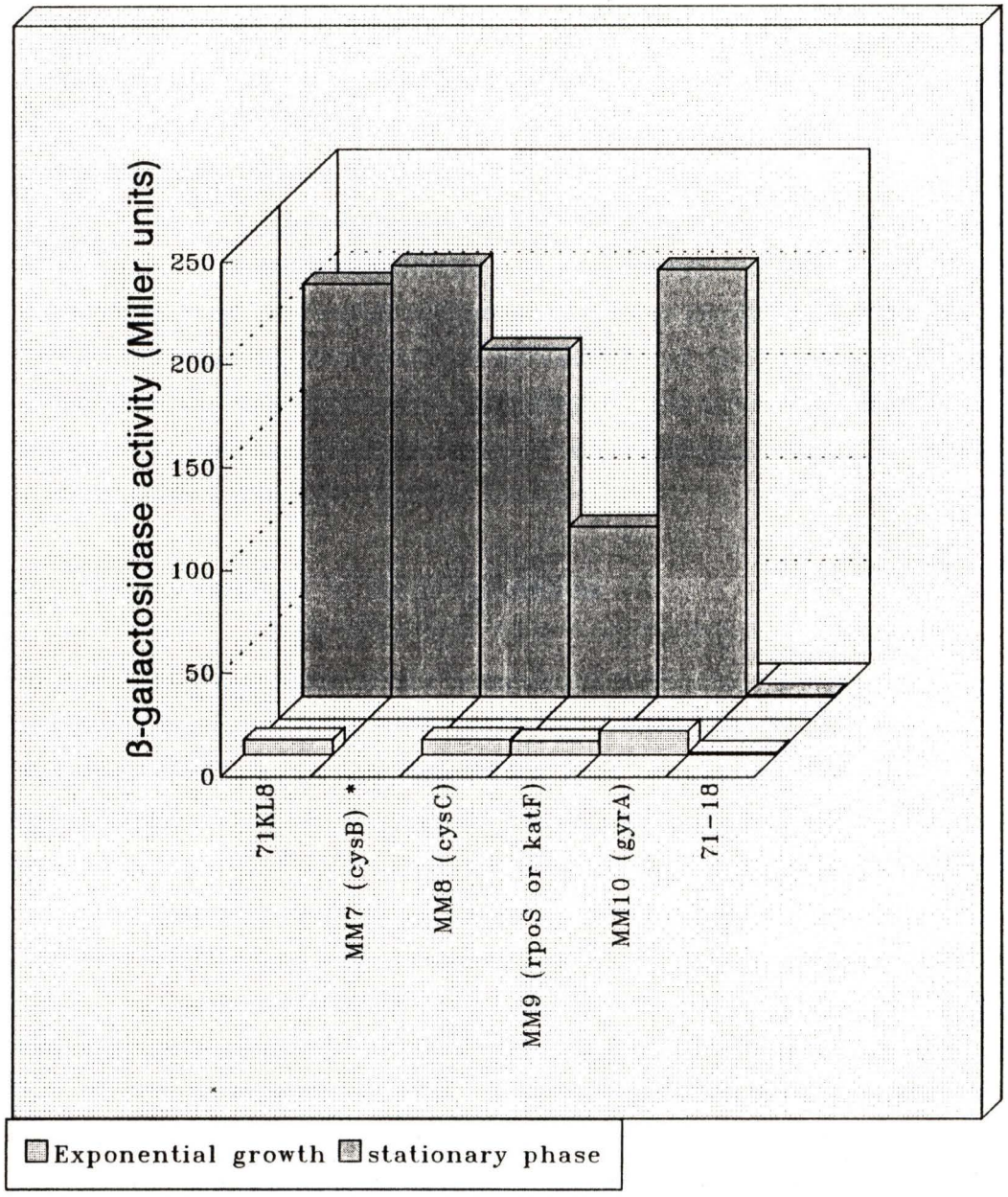


Figure 3.15. Effect of secondary mutations on β–galactosidase activity of 71KL8. Exponential and stationary phase β-galactosidase activity was measured for 71KL8 derivatives with *cysB*, *cysC*, *rpoS* or *gyrA* mutations. Each reading is the average of duplicate cultures, with all β-galactosidase assays done in duplicate. Cultures were purified by at least 10 generations of exponential growth prior to exponential phase determination. MM7 (*cysB*) was only assayed at stationary phase.

4.2. Activity in rich media (LB)

The β-galactosidase activity of 71KL8 in rich media has been described above. Adding NaCl to 0.35M resulted in no significent change in expression during growth, but activity was doubled during stationary phase. The effect of addition of complementing plasmid pAD300 was also observed in LB, although only during stationary phase. Activity was decreased after both one and two days, although this decrease was not large. As *cysQ* is not required during anaerobic growth (Neuwald *et al.* 1992), the effect of anaerobiosis was also assessed. Test tubes were filled with inoculated LB and then sealed with parafilm, prior to incubation without shaking at 37°C. Similar conditions were shown by Volkert *et al.* (1989) to result in induction of anaerobically controlled genes. Stationary, but not exponential, β-galactosidase activity was observed to be decreased in anaerobic conditions. All results are summarised in figure 3.14.

4.3. Genes involved in *cysQ* regulation

In order to characterise the genetic determinants responsible for regulation of *cysQ*, a number of candidate genes were transduced to 71KL8 by P1 transduction. Two mutations effecting cysteine biosynthesis were introduced. The first, *cysB* (the transactivator of cysteine biosynthesis), was transduced in two steps; *trpB83::*Tn*10* (a selectible marker genetically linked to *cysB*) was introduced into *cysB* strain CB64. A lysate prepared on the resulting Tet^r strain, MM5, was used to transduce 71KL8 to Tet^r. Tet^r transductants were screened for cysteine and tryptophan auxotrophy, with one cys^- trp^- transductant being designated MM7. A *cysC* mutant of 71KL8, MM8, was obtained by transduction from N3002, a *cysC-95::*Tn*10* strain, with selection for Tet^r; all Tet^r transductants were cys^-. MM9, a *rpoS* (*katF*) strain deficient in σ_S, the stationary phase σ factor, was constructed by transduction into MM8 (*cysC*). The rationale was that *cysC* is genetically linked to *rpoS* and could, therefore, be used as a marker for transduction. As it was thought to be important to exclude *cysC* fron the genetic background, MM8 was transduced by a lysate of rpoS (*katF3*) strain UM 56-64, with selection on M63 plates for prototrophy. Of the several hundred prototrophs obtained by transduction, 32 were tested for Tet^S and catalase activity. *RpoS* are $catalase^-$, which can be tested by dropping H_20_2 onto colonies, scoring for the release of O_2 due to catalase action. Oxygen release from $rpoS^+$ strains causes profuse bubbling, which is absent in *rpoS* strains. All 32 transductants were Tet^S and 16 were *rpoS*, giving a cotransduction linkage of 50% which agrees well with published linkage of 45% (Bohannon *et al.* 1991). One of the 16 $cysC^+$, *rpoS* transductants

was designated MM9. The final strain constructed, MM10, was a *gyrA* mutant of 71KL8, obtained by transduction from KL166 (*gyrA13*) with selection for Nal^r. It was also hoped to determine the effect of cya or crp, fnr, dye and hns mutations, however I was not able to obtain the donor strains in time to do this.

4.4. Activity of 71KL8 with secondary mutations

The β-galactosidase activities of MM8 (*cysC*), MM9 (*rpoS*) and MM10 (*gyrA*) were determined in both exponential and stationary phase, but only stationary phase activity was determined for MM7 (*cysB*). All results are summarised in figure 3.15. During exponential growth activity was not significently altered in any strains, although the low β-galactosidase activities may mask differences. No changes in stationary phase expression levels were observed for MM7 or MM10, and a moderate decrease in expression was observed for MM8. A far larger 2.5 fold decrease, from 201 to 83 Miller units, was observed for MM9, the *rpoS* mutant of 71KL8 deficient in the stationary phase σ factor, σ^S. It was also observed that the growth of MM9 was retarded on minimal media, although time did not permit clarification of whether this growth deficit was caused by decreased expression of *cysQ*.

4.5. Determination of *cysQ* promoter

Subcloning of *cysQ::*Tn*10-LK* containing plasmids pMM80 and pMM100 was done in an attempt to confirm that both fusions were translated from the same promoter and as a preliminary step in determining the sequence necessary for initiation of transcription and translation of *cysQ*. To eliminate the *SalI-NcoI* fragment containing the entire 5' end of *cysQ*, as well as all upstream sequences, pMM80 and pMM100 were digested wtih *SmaI* and *NcoI*. The cohesive ends of the *NcoI* site were filled, and the plasmids were then religated in a large volume. Transformants of DH5α that had lost the *SalI-NcoI* fragment were called pMM81 and pMM101 respectively. Surprisingly both pMM81 and pMM101 retained strong β-galactosidase activity, but time did not permit any clarification of this observation.

4.6. Stability of $CysQ_8$-lacZ fusion protein

Results from β-galactosidase assays suggested that the $CysQ_8$-LacZ fusion protein was very stable, with β-galactosidase activity of 71KL8 remaining approximately constant after several days, despite viable cells decreasing to 10% of their original

number (results shown in figure 3.16). In addition, incubation of chloroform lysed cells in Z buffer confirmed there to be no decrease in β-galactosidase activity, even six hours after lysis. These results suggested that protein stability might be a factor in CysQ function. As a first step in clarifying the importance of protein stability, 71KL8 was grown to stationary phase in LB, and then chloramphenicol (to 30μg / ml), or chloroform (100μl), was added. The cultures were incubated for two days and the β-galactosidase activity was determined at intervals. Results compared with control non-treated 71KL8 are shown in table 3.2. Although preliminary, these results do suggest the $CysQ_8$-LacZ fusion protein to be very stable, with a half life in this determination of approximately four days.

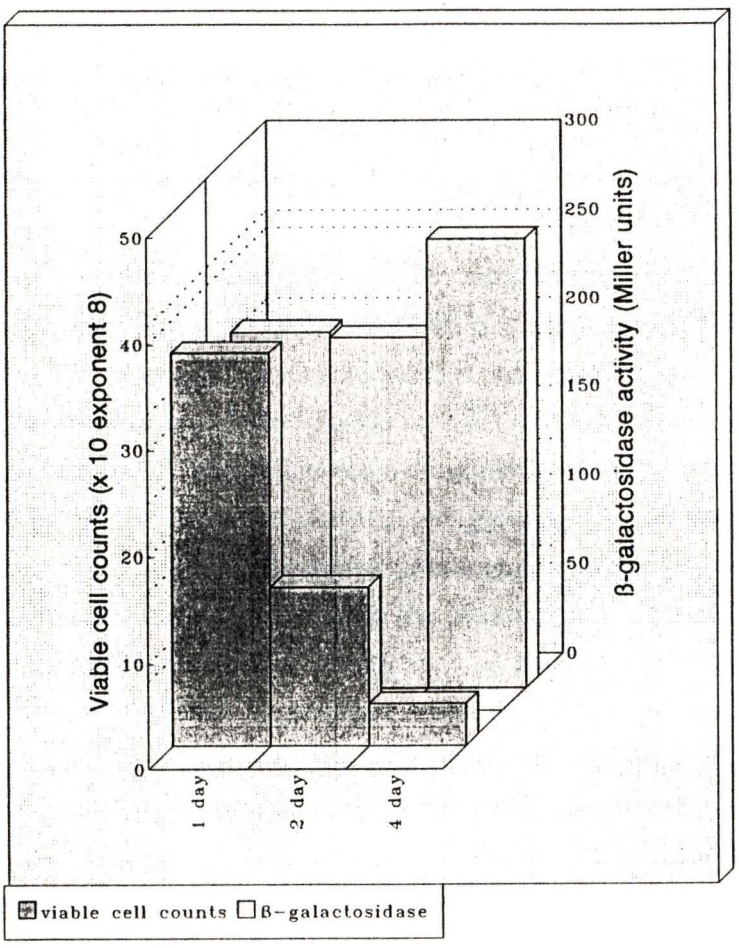


Figure 3.16. β-galactosidase activity versus viable cell counts for 71KL8. Duplicate cultures of 71KL8 were grown to saturation in rich media and left for 4 days incubating shaking at 37°C. Viable cell counts and β-galactosidase assays were done at intervals.

Table 3.2. Stability of $CysQ_8$-LacZ fusion protein. β-galactosidase activity of chloramphenicol or chloroform treated duplicate stationary phase cultures of 71KL8 was measured over a 48 hour interval and compared with non-treated 71KL8 cultures. All β-galactosidase activities are in Miller Units.

		Hours		
		7.5	24	48
β-galactosidase activity	chloramphenicol treated	235	204	143
	chloroform treated	205	195	150
	non-treated	201	201	198

---

5. CLASSIFICATION OF OTHER MUTANTS

5.1. Phenotype and complementation

AM77 was one of a group of nine osmosensitive mutations originally isolated by McLellan that had mini Tn*10kan* insertions within a 2.9kb *EcoRI* fragment. To determine whether the other eight mutations were at the same locus, AM77 and the other eight mutants were examined for growth on M63 and M63 + 0.35M NaCl, and on a range of other media. All mutants were osmosensitive and this osmosensitivity was not removed by glycine betaine or choline. All grew poorly compared with wild type W3110, even in the absence of osmotic stress, and growth of all mutants was restored by the addition of cysteine, in both the presence and absence of osmotic stress. Addition of methionine and thiosulphate also enhanced growth, though to variable degrees (see figure 3.4). In addition, when pAD300 was transformed into the nine strains, all but K41 and L16 were complemented. As K41 and L16 might have contained insertions in the portion of the 2.9kb *EcoRI* fragment not contained in pAD300, pAD109 was transformed into both. Plasmid pAD109 (figure 5.3), contains the complete 13kb region from K656, including the 2.9kb *EcoRI* fragment, and complements AM77. Neither K41 or L16 were complemented by pAD109. In combination these observations suggested three classes of mutants: AM77 and AD36 were in one class, characterised by complementation by pAD300 and supplementation by methionine; K41 and L16 constituted a second group, with both being supplemented by methionine, but neither complemented by pAD300. The third group contained the remaining 5 mutants, of which V77 was typical; all were complemented

by pAD300, and none were supplemented by methionine. This third group phenotypically resembled WKL10. Growth of AM77, AD36 and K41, as representatives of these three groups, is shown in figures 3.6 and 3.8, while the results of supplementation by reduced sulphur sources are summarised in figure 3.4.

5.2. Determination of insertion position

The phenotype of all eight mutants suggested them to be mutants, like AM77, of either *cysQ* or an adjacent gene of related function. To test this possibility PCR was used. Two oligonucleotide primers were designed, one binding to the sequence above *cysQ* and oriented to be amplified through *cysQ* (*cysQ* primer), and the other to the IS10R sequence of any mini Tn*10* and oriented outwards (Tn*10*-IS10R primer). The oligonucleotide sequences are shown below, while a schematic of the binding sites and PCR products is shown in figure 3.17.

***cysQ* primer**	5'- GATGTGTATCCACCTTAACT-3'
Tn*10*-IS10R primer	5'-CGTCTTACTTATAGAACAGT-3'

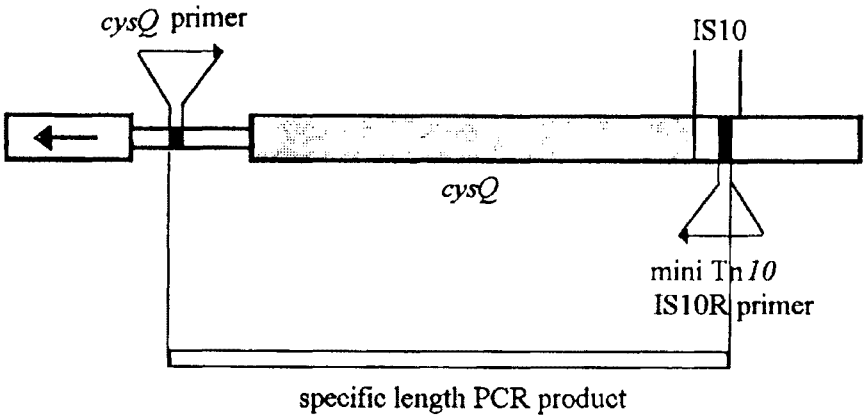
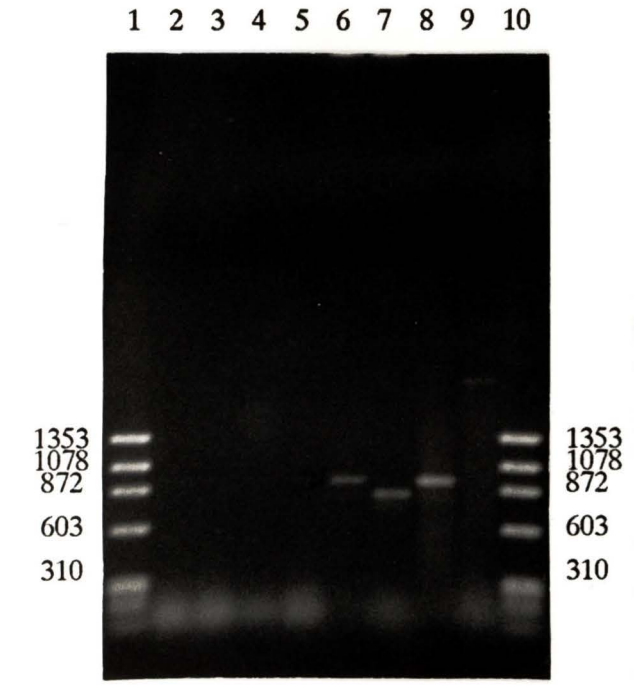


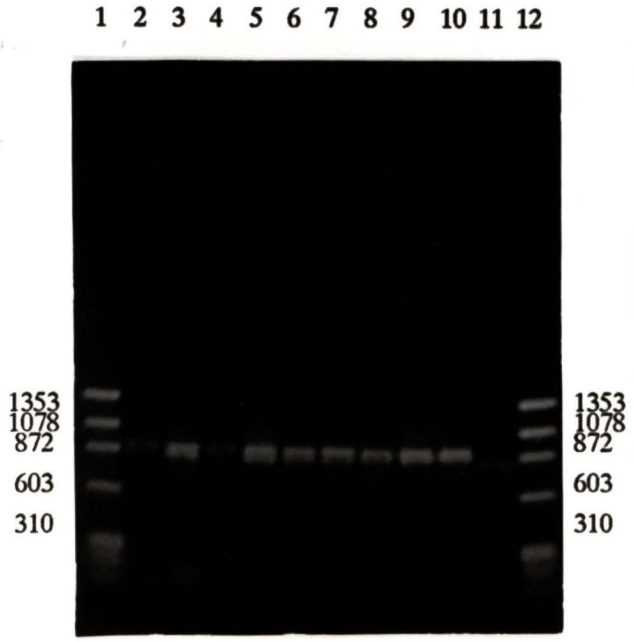
Figure 3.17. Schematic of PCR mapping of chromosomal mini Tn*10* insertions.

Figures 3.18A and B. PCR fragments from chromosomal mini Tn*10* insertions in, or downstream of, *cysQ*. The fragment from WCH7 is indicated by an arrow.

a)
1. ØX174 *HaeIII* digest
2. No template DNA
3. W3110
4. AM77 (no primer 1)
5. AM77 (no primer 2)
6. AM77
7. WKL10
8. WKL8
9. WCH7
10. ØX174 *HaeIII* digest

b)
1. ØX174 *HaeIII* digest
2. AM77
3. A26
4. AD36
5. K41
6. L16
7. H8
8. T4
9. V77
10. V62
11. WKL10
12. ØX174 *HaeIII* digest





Figures 3.18 A and B. PCR fragments from chromosomal mini Tn*10* insertions.

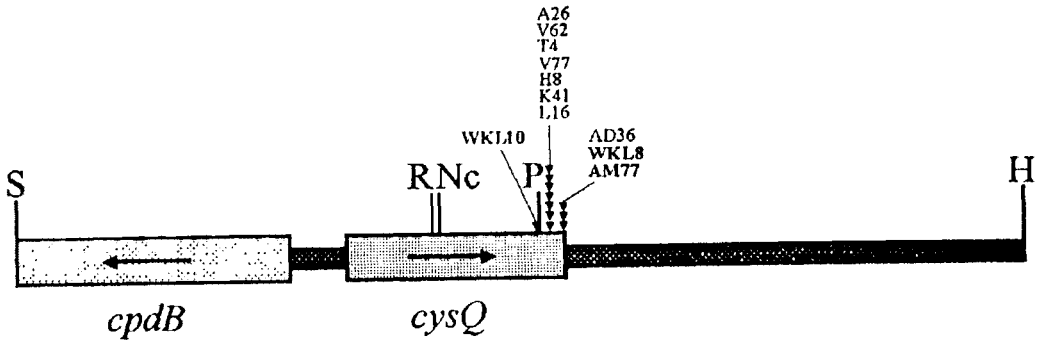


Figure 3.19. Insertion positions of the nine original mutants determined by PCR mapping. WKL8, WKL10 are also included to enable comparison.

It was expected that a PCR product would only be obtained if a mini Tn*10* insertion was present, and that the length of this fragment would enable mapping of the transposon insertion position. Amplification of the expected size products from plasmid and chromosomal insertions of *otsC::*Tn*10kan*, *cysQ10::*Tn*10-LK* *cysQ8::*Tn*10-LK* and *CH7::*Tn*10cam* confirmed that this method gave the correct sized PCR products for insertions up to at least 2.2kb from the *cysQ* primer (figure 3.18A), but no product was obtained from W3110 or 71-18, or if either primer was omitted.

The results from PCR are shown in figure 3.18B. PCR products were generated for all eight mutants, confirming all mutants to have insertions in the region of *cysQ*. Furthermore, all insertions were found to be very close to that of AM77. The insertion positions were further mapped by *NcoI* or *PstI* digestion of the PCR product, and are shown in figure 3.19.

5.3. Characterisation of K41 and L16

K41 and L16 were shown by PCR to be *cysQ::*Tn*10kan* yet they were not complemented for growth by pAD300. McLellan's original selection for osmosensitive mutants included two purifications of single colonies on M63. This suggested that K41 and L16 might have acquired secondary mutations in the cysteine biosynthetic pathway as *cysQ* mutants are known to accumulate suppressor mutations with high frequency (Neuwald *et al.* 1992). To examine this possibility I rescued the *cysQ::*Tn*10kan* insertions from K41 and L16 by λ K656 transduction as described in

Figure 3.20. Stationary phase survival. A comparison of viable cell counts for WKL10 and W3110 over 10 days. W3110 and WKL10 were grown to saturation in LB media, and then left for 10 days. Aliquots were removed each day for viable cell counts. Results are the average of 3 independent cultures for each strain.

section 2.3 of this chapter. K656 lysates prepared on K41 and L16 were used to transduce W3110. Six transductants from each were streaked on M63 and all were found to be auxotrophic. Addition of either cysteine or thiosulphate restored growth for all transductants. One transductant from K41 was designated MM17 and one from L16, MM16. PCR mapping of MM16 and MM17 confirmed both to have acquired *cysQ::*Tn*10* insertions in the expected positions. MM16 and MM17 were transformed with pAD300, which complemented both for growth on M63 and on M63 + 0.35M NaCl. The growth of K41 and derivatives are shown in figure 3.8. Growth of L16 and derivatives were qualitatively identical. For both MM16 and MM17 growth was substantially retarded compared with their parent strains. Neither MM16 or MM17 showed substantial supplementation by methionine, whereas both K41 and L16 were supplemented by methionine to wild type growth levels. By contrast, growth of both MM16 and MM17 was restored to wild type by addition of thiosulphate in conditions of osmotic stress, and in the presence of pAD300 whereas K41 and L16 were not. It was also noted that K41 and L16 did not accumulate secondary suppressor mutations, whereas the remaining seven insertions did.

6. STATIONARY PHASE SURVIVAL

As *cysQ::lacZ* fusions were maximally expressed in stationary phase it was decided to examine whether *cysQ* was necessary for stationary phase survival. Survival of WKL10 and W3110 in non-selective rich media (LB) was measured by comparing viable cell counts over a period of 10 days. To examine whether WKL10 had accumulated suppressor mutations (see below, section 7) during stationary phase, single colonies were streaked on M63 with WKL10 and W3110 as controls. Retention of the mini Tn*10-LK* was also examined by checking single colonies for kanamycin resistance. Results for survival are shown in figure 3.20. WKL10 survived as well as W3110 and did not accumulate secondary mutations and kanamycin resistance was retained.

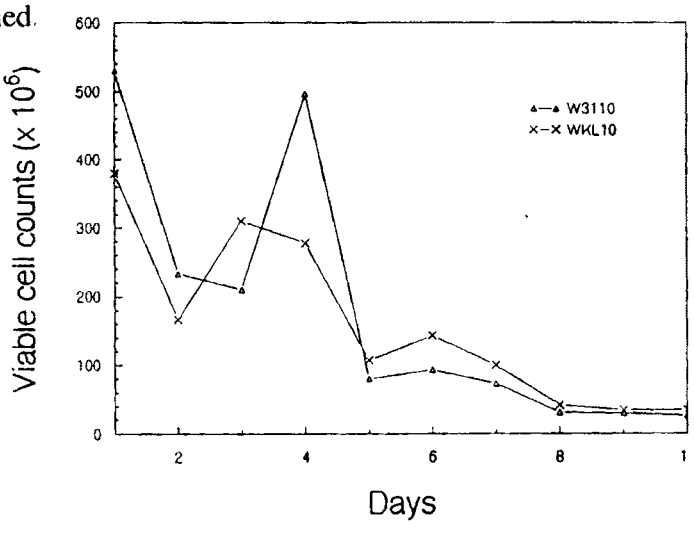


Figure 3.20. Stationary phase survival of WKL10 and W3110.

Figure 3.21. Growth of revertants of WKL10 compared with WKL10 and W3110. M63 plates were incubated for two days.

1. W3110
2. revertant 4
3. revertant 3
4. revertant 2
5. revertant 1
6. WKL10

Figure 3.22. Reversion during exponential growth in minimal media. Growth on M63 of single colonies isolated from WKL10 which had been grown to saturation in M63 broth. Ten colonies from each of three independent cultures are shown (delineated by blue lines).

1. W3110 (wild type)
2. WKL10 (*cysQ*$_{10}$::Tn*10-LK*)
3. a revertant
4. a culture in which all ten single colonies were revertants

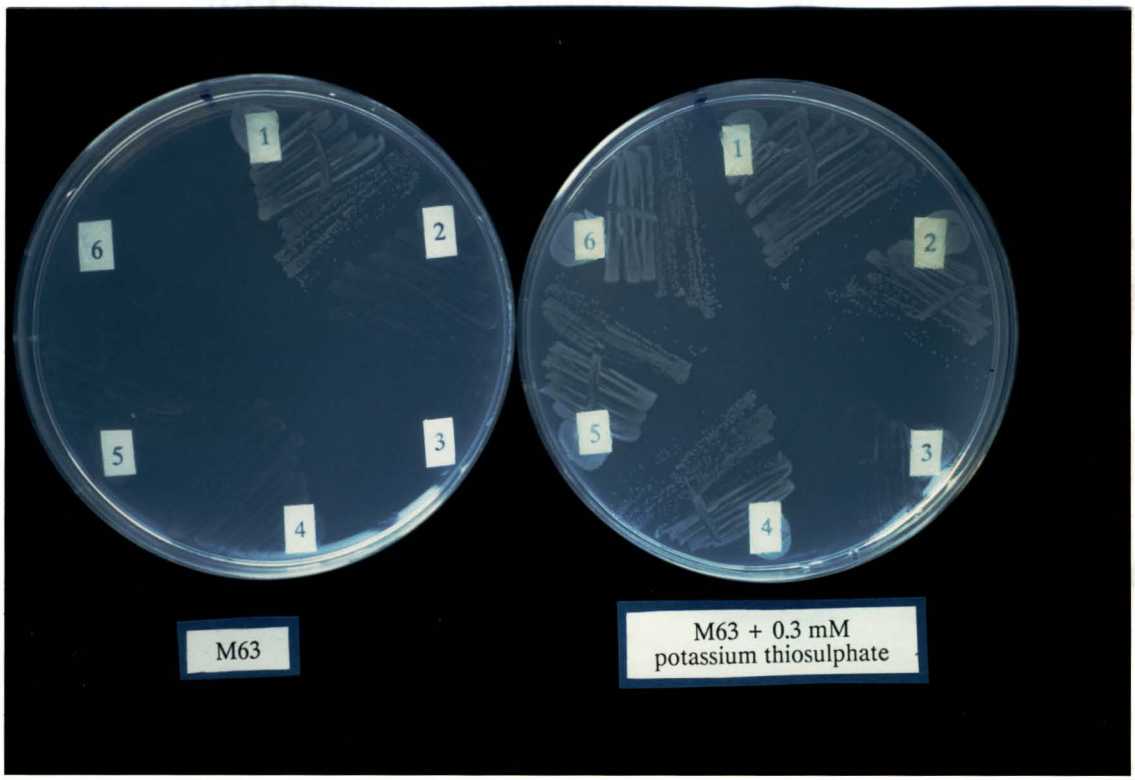


Figure 3.21. Growth of revertants of WKL10.

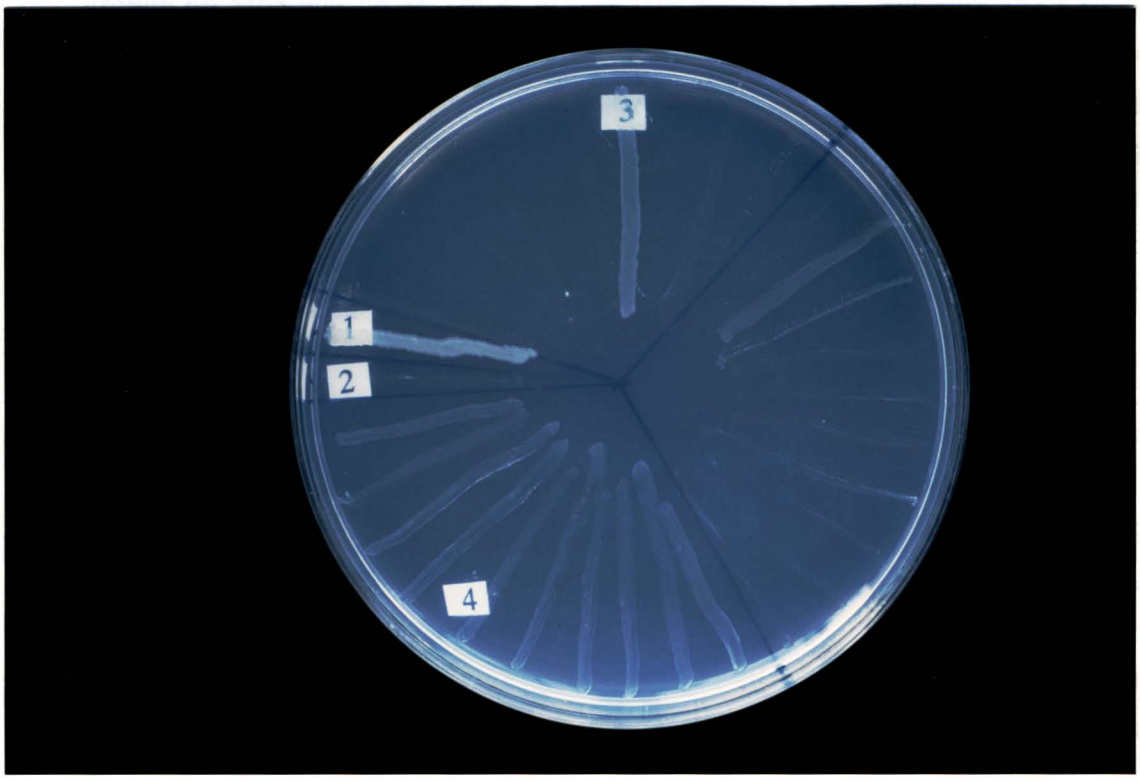


Figure 3.22. Reversion during exponential growth in minimal media.

7. SUPPRESSOR MUTATIONS ACQUIRED BY *CYSQ* MUTANTS

Reversion mutants of both AM77 and WKL10 were observed during growth on most minimal media used in this study. As observed for AM77 in section 3.11, these revertants were heterogeneous in size and visible as distinct colonies against a lawn of AM77 growth. WKL10 revertants were also visible as distinct colonies but their frequency was less than that of AM77. The frequency of reversion was confirmed by growing overnight cultures of AM77 or WKL10, resuspending these in minimal salts and then spreading dilutions on M63 plates. Numbers of revertant colonies were counted, as were the number of cells plated, and the reversion frequencies were calculated. The frequency of reversion in AM77 was calculated as 1.7×10^{-3} while for WKL10 the frequency was 1.1×10^{-4}. Both figures are the average of three independent cultures.

Considerable heterogeneity was observed both in the extent of increase in growth caused by secondary mutations, and in supplementation by different sulphur sources. Four independent revertant colonies of WKL10 and AM77 were isolated and their growth on M63 was compared to W3110 (see figure 3.21). For both WKL10 and AM77 three of the four revertants were noticeably more able to grow in the absence of cysteine or methionine, while the presence of thiosulphate restored growth to wild type levels. Two revertants from each strain were shown by PCR mapping to retain their mini Tn*10*, with the PCR fragment size remaining unchanged. Characterisation of mutants of 71KL10 containing suppressor mutations confirmed that *lacZ* activity was retained and that pAD300 was unable to complement the mutant strains to wild type growth.

7.1. Reversion in liquid cultures

As both AM77 and WKL10 developed frequent secondary mutations on solid media, the presence of mutations in liquid media was examined. Single colonies from WKL10 and AM77 grown in a range of media were isolated by non selective streaking on LB. Single colonies were then examined for growth on minimal and kanamycin plates, to determine whether reversion or mini Tn*10* excision had occurred. Results are shown in table 3.3. The proportion of colonies bearing

Table 3.3. Reversion frequency in liquid cultures. Single colonies were isolated from M63 or M9 cultures of either 71KL10, WKL10, MM6, or AM77. Ten or twenty single colonies from each culture were streaked on minimal media and compared to the parent mutant. Results show the percentage of revertents in an individual culture. No revertents were observed prior to growth in minimal media.

	Percentage of revertents for independent cultures					
71KL10 M63	65	45	45	10	65	45
WKL10 M9	10	20	100	90	-	-
AM77 M9	0	0	50	0	-	-
MM6	0	70	-	-	-	-

suppressor mutations was highly variable, differing greatly for individual cultures. The occurrence of suppressor mutations appeared to be suppressed by the addition of reduced sulphur sources; only in one instance were any suppressor mutations observed in a culture supplied with any reduced sulphur source. In all cases Kan^r was retained despite there being no selection for it.

7.2. Stability of 71KL8

71KL8 proved to be unstable if left on plates for long periods. All surviving colonies from a two-month-old plate were auxotrophs which were complemented by addition of cysteine. All retained Kan^r and *lacZ*, and PCR mapping of two of them confirmed both to retain a mini Tn*10* in the expected position with no sign of visible deletions. Unfortunately shortage of time precluded further study of the basis of this instability.

CHAPTER FOUR

DISCUSSION

In this study, I have been involved in characterising AM77, a mini Tn*10* mutant of *E. coli* initially thought to be involved in trehalose synthesis. AM77 was originally selected as a mutant that was defective in osmoregulation in the absence of external osmoprotectants such as betaine. The mutation was further characterised by McLellan (1992) as being deficient in osmoregulatory trehalose synthesis for three reasons: failure to grow on defined media with 0.35M NaCl added; decreased internal trehalose content; and aberrent growth on minimal media with trehalose as a carbon source. On this basis, McLellan concluded that AM77 was mutated in *otsC*, a fourth gene, other than *otsA*, *otsB* and *galU*, which was required for osmoregulatory trehalose synthesis. The mini Tn*10* insertion of AM77, *otsC::*Tn*10kan*, was physically mapped to a 1.4kb region below *amtA*, at 95.7 minutes on the *E. coli* chromosome, and was observed to grow normally on limited nitrogen, conditions in which *amtA* mutants do not grow. However, during this study I have shown by complementation, amino acid supplementation and eventually by sequencing, that the Tn*10kan* insertion of AM77 is within *amtA*, which has subsequently been found to be involved in cysteine biosynthesis, and has been renamed *cysQ*.

1. AM77 AND THE OTHER EIGHT MUTANTS ALL CONTAIN MINI TN*10* INSERTIONS IN *CYSQ*

1.1. Localisation of the AM77 mini Tn*10* insertion

Nine osmosensitive mutants previously isolated by McLellan (1992) appeared to contain single copy chromosomal insertions of mini Tn*10* into a common 3kb *EcoRI* chromosomal fragment. The insertion of one mutant, AM77, was mapped to 95.7 minutes on the *E. coli* chromosome, immediately to 3' of *cysQ*. The present study confirmed that AM77 and the remaining eight mutants, along with other mini Tn*10* insertions of interest, all mapped to this location on three grounds: all mutants yielded PCR products specific to mini Tn*10* insertions in, or downstream of, *cysQ*, but the parent strain did not; the mutations caused by the mini Tn*10* insertion could be complemented by cloned wild type DNA from this region; insertions could be transduced by K656, a λ phage which contained DNA homologous to this region, but not by K446 which contained non homologous DNA.

1.2. AM77 is a *cysQ* mutant.

AM77 was proven in this study to be a mutant of *cysQ* on four grounds, these being: AM77 has a similar phenotype to a known *cysQ* mutant, WKL10; AM77 is complemented by the same fragment of wild type DNA that complements WKL10; only mini Tn*10* insertions in pAD300 that map to within *cysQ* fail to complement AM77; and sequencing showed *otsC::*Tn*10kan*, the insertion in AM77, to be within *cysQ*.

In this study it was observed that AM77 grew poorly in the both the presence and absence of osmotic stress (bradytrophic), with addition of cysteine restoring normal growth in both the presence and absence of added NaCl. Growth of AM77 was similar to that of WKL10, a known *cysQ* mutant, and to that described for *cysQ* mutants (Neuwald *et al.* 1992). Both AM77 and WKL10 were complemented by plasmid pAD300, but saturation mutants of pAD300 (McLellan 1992) failed to complement AM77 on M63 + 0.35M NaCl. In the absence of osmotic stress only those saturation mutants with insertions in *cysQ* failed to complement AM77. The region of pAD300 required for complementation of AM77 was also defined by *BAL31* digestion and subcloning, and it was found that the complete *cysQ* sequence, but not the sequence below *cysQ*, was required for complementation of both AM77 and WKL10, in both the presence and absence of added NaCl. It was also found that a subclone which removed the 5' end of *cysQ*, pMM450, would not complement AM77.

The results obtained were not consistent with AM77 requiring any sequence below *cysQ* for complementation, and did not agree with those of McLellan (1992), who found a 1.4kb region below *amtA* (*cysQ*) to be required for complementation of the osmosensitive phenotype of *otsC*. The results do, however, agree with those of Fabiny *et al.* (1991) and Neuwald *et al.* (1992), who found a fragment, which included the whole *cysQ* sequence, to be required for complementation of *cysQ* or *amtA* mutants. It was noted that McLellan had also observed deletions of the promoter region of *cysQ* (in pAD504 and pAD505) to prevent complementation, and that McLellan had incorrectly mapped *amtA* (*cysQ*) with respect to its internal *EcoRI* and *PstI* sites, which resulted in transposon insertion sites, found in this study to be within *cysQ*, being mapped as below *cysQ*.

To clarify whether sequences below *cysQ* were required for complementation of AM77, the insertions from three saturation mutants were transduced into the chromosome. WKL10, which had a Tn*10* insertion in *cysQ*, had a similar phenotype to AM77; it failed to grow on M63 + 0.35M NaCl, and grew even more poorly than AM77 on M63 alone. By contrast, strains WKL8 and WCH7 (from mutants pKL8 and pCH7, both of which failed to complement AM77 only in the presence of 0.35M NaCl) were both prototrophs in all conditions tested. This suggested that the observed lack of complementation of saturation mutants at 0.35M NaCl was not a specific effect caused by the position of the mini Tn*10* insertion, but was a non specific effect caused by the presence of mini Tn*10*, combined with the high copy number of the pBR322 based pAD300. Loss of complementation was observed for two different mini Tn*10* types, therefore, complementation was not antibiotic resistance dependent. Unfortunately no negative control insertions which retained complementation had been isolated, and in retrospect I would have repeated saturation mutagenesis for this purpose.

1.2.a. Sequencing of the insertion sites.

Sequencing of *otsC::*Tn*10kan* , *cysQ$_8$::*Tn*10-LK* and *cysQ$_{10}$::*Tn*10-LK* proved all three insertions to be within the published sequence for *cysQ* (Fabiny *et al.* 1991). Although confirming that the mutation in AM77 is caused by an insertion in *cysQ*, these results also showed the insertion *cysQ$_8$::*Tn*10*-LK to be in an identical position; both were three codons within the 3' end of *cysQ* and in frame. The phenotype of the two insertions, when present in single copy chromosomal insertions, was, however, observed to be quite different; whereas AM77 exhibited retarded growth in both the presence and absence of osmotic stress, growth of WKL8 appeared to be wild type in both conditions. These observations can be explained by the type and orientation of the markers present in the respective transposons. The *lacZ* gene of *cysQ$_8$::*Tn*10-LK* is fused to *cysQ*, with transcription under the control of the *cysQ* promoter, whereas *otsC::*Tn*10kan* contains a Kan^r marker, which is oriented so that any transcriptional over-run would cause transcription through *cysQ*, and in the opposite direction to *cysQ* transcription (see pAD77 figure 5.4). Neuwald *et al.* (1992) isolated a *cysQ* mutant, DBan41, which contained a Tn*5tac1* insertion two codons within the 3' end of *cysQ* and which was prototrophic except when IPTG was added. IPTG induced the Tn*5tac1* promoter, which was transcibed

through *cysQ* and towards the *cysQ* promoter, thereby causing cysteine auxotrophy. As mini Tn*10* can cause polar effects (Kleckner *et al.* 1991), this could explain the observed differences between the phenotype of AM77 and WKL8.

1.3. All mutants isolated by McLellan were *cysQ* mutants

Seven of the remaining eight mutants isolated by McLellan, other than AD36, were shown to be within *cysQ* by PCR mapping. AD36 could not be positively mapped to *cysQ* on the basis of PCR fragment length, but, in combination with its observed phenotype, there is strong evidence for AD36 also being a *cysQ* mutant. An obvious relationship between insertion position and phenotype was observed for all *cysQ* mutants; as mutations got closer to the end of *cysQ*, the growth deficiency decreased. AD36 < AM77 < all remaining insertions (including WKL10). On this basis both AD36 and AM77 should be classified as $cysQ^{Reduced}$, while all others should be $cysQ^{-}$. In justifying the assignment of a $cysQ^{-}$ genotype to strains that are still able to grow even in the absence of cysteine, I noted that slow growth was observed by Neuwald *et al.* (1992), even for strains with large deletions in *cysQ*.

It is unknown whether the remaining seven insertions, other than AM77 and AD36, are inserted in the same position or orientation, or even whether they are truly independent isolates or if they are clonal, although they were isolated from several independent mutagenesis experiments (McLellan 1992). No differences between insertions could be determined from restriction digests of PCR products, with all mapping to approximately 50 bp inside the 3' end of *cysQ*. To clarify the positions of these unknown insertions a more accurate PCR based mapping regime could be used, based on one sided PCR amplification of the *PstI* digested fragments using only the Tn*10*-IS10R primer and radiolabelled ATP. This would yield fragments that could be seperated on a polyacrylamide sequencing gel and accurately mapped by comparison to a sequencing ladder. Alternatively the insertion position could be sequenced directly from the PCR products.

The positions of all mini Tn*10* insertions characterised in this study suggest the presence of a Tn*10* hot spot in this region (see figures 4.1A and 3.14). Wild type Tn*10* has an insertion specificity for the consensus 5'-NGCTNAGCN-3', although there is a large degree of variation (Kleckner *et al.* 1991). Of nineteen

mini Tn*10* insertions examined in this study, seventeen were within a 150 bp fragment, effectively the last 150 bp of *cysQ*. An examination of the *cysQ* sequence did not reveal any obvious Tn*10* consensus sequences, although the sequence immediately below *cysQ* was AT rich, which makes it likely to be "cold" for insertions (Kleckner *et al.* 1991).

The results of mapping of the insertion positions, along with complementation by deletion subclones, confirm all but the last few residues of *cysQ* to be required for activity. Insertions up to 9 bp within *cysQ* have normal or almost normal activity, whereas insertions such as that of WKL10 and V77, which are 120 bp and approximately 50 bp within the 3' end of *cysQ* respectively, have no activity.

2. *CYSQ* IS NECESSARY FOR CYSTEINE BIOSYNTHESIS.

2.1. Ammonium methylammonium uptake

Mutants of *cysQ* have been characterised previously, first as deficient in ammonium methylammonium uptake (Jayakumar *et al.* 1989), and later as cysteine requiring bradytrophs (Neuwald *et al.* 1992). Both phenotypes were examined in this study. Neither AM77 nor WKL10 were found to grow on M9 media with limited nitrogen, in agreement with observations of *amtA* (Jayakumar *et al.* 1989) and *cysQ* strains (Neuwald *et al.* 1992). All mutants were observed to grow as wild type when cysteine was added, once again in agreement with Neuwald *et al.* (1992). Examination of the results of Jayakumar *et al.* (1989) suggests that growth of their mutant, AJ2653, was retarded even in the absence of limiting nitrogen, supporting the hypothesis that the *amtA* locus has a role other than ammonium methylammonium uptake. McLellan (1992), however, found AM77 to be *amtA*$^+$, which may have been because he used M63 rather than M9 media, as was used by Jayakumar and coworkers (1989) and in this study. Alternatively McLellan's observations may have been because AM77 is, as already discussed, *cysQ*^{Reduced}. In combination these factors could have resulted in AM77 growing enough to be considered *amtA* $^+$.

2.2. Sulphur sources

The mutant phenotypes observed for all *cysQ* mutants classified in this study were suppressed by addition of cysteine, confirming the relationship of *cysQ* to cysteine biosynthesis, although a number of complicating factors were observed. Cysteine itself proved to be toxic to cells, causing fluctuating growth lags. This effect has been reported previously (Qureshi *et al.* 1975, Harris. 1981, Sorensen and Pederson 1991), and is thought to be caused by inhibition of threonine deaminase, resulting in isoleucine starvation. For this reason cysteine could not be said to supplement *cysQ* mutants to wild type growth, even though it was apparent that cysteine caused suppression of the mutant phenotype. Thiosulphate supplementation did, however, allow wild type growth, except for K41 and L16 (which are discussed below), proving *cysQ* to be cysteine biosynthesis related.

Neuwald *et al.* (1992), found the growth of *cysQ* mutants to be restored by the addition of sulphite. Some growth enhancement was observed in this study, but substantially less than for cysteine. As observed by Postgate (1963), sulphite is very unstable in physiological conditions, so these observations are perhaps not surprising. Conditions used in this study were the same as those of Neuwald *et al.* (1992), with fresh solutions of 0.3mM sulphite being used for each experiment. The observed difference is, therefore, difficult to explain, although one possibility is that our stocks of (somewhat old) potassium sulphite might have spontaneously oxidised to sulphate, which has been observed to occur readily (Postgate 1963).

Addition of methionine resulted in an interesting phenotype, for it allowed AM77, but not WKL10, to grow as wild type, but only on M63; in the presence of 0.35M NaCl, AM77 was unable to grow. Methionine can not act as a source of reduced sulphur, but it can reduce the cysteine requirements of the cell by approximately half (Qureshi *et al.* 1975, Kredich 1987). This suggests that methionine returns AM77 to wild type growth by sparing cysteine, thereby reducing cysteine requirements, and allowing AM77 to grow. This hypothesis is supported by the *cysQ* ^{reduced} phenotype of AM77. For methionine sparing of cysteine to occur an active cysteine biosynthetic pathway is required (Qureshi *et al.* 1975). By extension, the failure of methionine to substantially affect the growth of WKL10 implies a far greater deficiency in cysteine biosynthesis.

The implications of methionine failing to complement AM77 in M63 + 0.35M NaCl are discussed in section 8.

Similar observations of growth enhancement caused by methionine have been made by Qureshi *et al.* (1975) and Russel *et al.* (1990). Russel *et al.* observed two *cysA* mutants of *E. coli* to grow when supplemented with methionine. Qureshi *et al.* reported a number of *S. typhimurium* mutants, classified as Cym, which had mutations in *cysA, cysC, cysD, cysG, cysH, cysI* or *cysJ*, and which were complemented to varying degrees by addition of both methionine and cysteine; null alleles of these same genes were unable to grow on methionine as a sulphur source. The growth curves of the Cym mutants were very similar to those observed for AM77 in this study. Qureshi *et al.* showed that methionine was unable to act as a source of reduced sulphur, and that cysteine biosynthetic enzymes were required for growth on methionine to occur, but not when cysteine was supplied, confirming that methionine was not being converted back to cysteine by a previously unknown pathway. An obvious conclusion from these results is that the mutations in question resulted in partially active cysteine biosynthetic enzymes, with addition of methionine sparing cysteine and thereby restoring function.

A number of variables were encountered in this study which complicated the interpretation of results obtained, including, as already mentioned, stability of reduced sulphur sources and the lack of a sulphur free media. In this study first M63 and then M9 media was used (with no added sulphur source), but even M9 contained enough sulphur as contaminants to support growth to greater than half that reached with no limitation on sulphur. Attempts to obtain sulphur free media by growing *E. coli* to saturation and then purifying the media, were also unsuccessful, with the resulting filtrate unable to support bacterial growth, even when additional glucose and sulphate were added. For similar reasons it was impossible to obtain sulphur free solid media because of Sulphur in the agar.

3. *CYSQ* IS NOT REQUIRED FOR TREHALOSE SYNTHESIS OR CATABOLISM

It was hypothesised by McLellan (1992) that the osmosensitivity observed for AM77 was caused by a lesion in osmoregulatory trehalose synthesis. However, in this study it was observed that growth of AM77 was enhanced by neither glycine betaine nor its precursor choline, and that the addition of cysteine or

thiosulphate restored growth. Cysteine, thiosulphate, sulphate and other forms of sulphur, other than glutathione, are not accumulated in the cell during osmotic stress (Cayley *et al.* 1991,1992). Glutathione, although a putative osmoprotectant, is only required in the absence of trehalose (McLaggen *et al.* 1990). If the addition of cysteine or thiosulphate was necessary for synthesis of glutathione as an osmoprotectant, it would be expected that glycine betaine would be able to supply this osmoprotectant requirement. The results obtained in this study are not consistent with the *cysQ* product being required for synthesis of an osmoprotectant.

AM77 was observed to be sensitive to externally supplied trehalose, with growth retarded on a range of media with trehalose as the carbon source. McLellan (1992), suggested that this might result from AM77 exerting a polar effect on *treC*, which maps to 96.5 minutes (Boos *et al.* 1990), and with *treC* being transcribed on a single mRNA from the *otsC* promoter. *TreC* mutants can give a trehalose-sensitive phenotype in a *treA*$^-$ background, with the evidence for AM77 being *treA* being its poor growth on M63 + 0.25M NaCl + trehalose. The results obtained in this study do not support this hypothesis. In all instances retarded growth with trehalose as a carbon source was suppressed by the addition of either cysteine or thiosulphate. In addition decreased growth, which was also reversed by the addition of cysteine or thiosulphate, was observed with maltose as the carbon source (results not shown). These observations imply that it is not the carbon source, but the absence of a reduced sulphur source, that is causing the lack of growth observed for AM77. In addition, strains WCH7 and WKL8 were both prototrophic and able to utilise trehalose as a carbon source in all conditions. The prototrophy of these strains eliminates the possibility that transposon insertions in *cysQ* could be disrupting transcription of *treC*, as both strains have mini Tn*10* insertions that would disrupt the putative *otsC-treC* transcript.

The results in this study do not suggest any role for *cysQ* in osmoregulatory trehalose synthesis, although this possibility can not be completely excluded as the internal trehalose levels of AM77 have not been measured. All other observations that were attributed to defective trehalose metabolism can be satisfactorily explained by disruption of cysteine biosynthesis. It is, therefore, proposed that the *otsC* designation of AM77 be changed to *cysQ*. McLellan's finding of a 60% decrease in trehalose synthesis in AM77 were based on the data presented in table 1.1. It is notable that, while the pBR322-based pAD300

complements trehalose levels to wild type, pAD200, which contains the same DNA fragment in a pACYC184 vector and which should, therefore, be present in lower copy number than pAD300, results in a trehalose content of three times that of the wild type. To clarify whether *otsC* does have a role in osmoregulatory trehalose synthesis it would be desirable to repeat this internal trehalose assay, preferably without the high glycogen background of W3110. A means of doing this would be to transduce *otsC::*Tn*10kan* to a strain that does not overproduce glycogen, such as MC4100, thereby allowing the anthrone method of trehalose determination (Norris and Ribbons 1971) to be used.

4. MUTATIONS IN *CYSQ* ARE BOTH LEAKY AND UNSTABLE

Neuwald *et al.* (1992) observed *cysQ* mutants to accumulate secondary mutations with high frequency, which was confirmed in this study. Although they also reported a high incidence of secondary mutations on plates, they do not do so for liquid cultures. Rather, they found *cysQ* mutants to be "leaky auxotrophs", or bradytrophs, with single colonies isolated after growth in minimal media having the same phenotype as their parent strain. In this study the accumulation of secondary mutations in liquid cultures was examined for a large number of cultures, and in the presence of different reduced sulphur sources. The results obtained confirmed growth to occur in the absence of secondary mutations, in other words *cysQ* mutants were confirmed to be "leaky". However, it was also found that secondary mutations did occur with high but variable frequency, with the number of revertants in individual cultures varying from 0/10 to 10/10 of the single colonies tested. Thus it must be concluded that mutants of *cysQ* are both leaky and highly revertible. In addition the occurrence of suppressor mutations was observed to be repressed in the presence of reduced sulphur sources.

The secondary mutations observed had a heterogenous, graded effect on growth. Further examination of some of these revertants showed that resistance markers were retained, even without selective pressure, and revertants were heterogenous in size in the absence of reduced sulphur, but grew as wild type when cysteine was added (with the proviso about cysteine toxicity discussed above). The growth of mutants K41 and L16 was examined in more depth. Both grew better than their parent mutants on minimal media in the absence of a

reduced sulphur source, but neither was wild type. The original Tn*10kan* markers of both strains were rescued, resulting in strains with far greater growth deficiencies, confirming the presence of a secondary mutation unlinked to *cysQ*. Plasmid pAD300 was unable to complement either mutant, but it did complement both of the rescued strains, proving the secondary mutation to be dominant over the *cysQ* mutation. These results were in agreement with observation of suppressor mutations of *cysQ* by Neuwald *et al.* (1992).

Examination of the supplementation effects of various reduced sulphur sources also provided some interesting observations. Methionine restored the growth of both K41 and L16 in both the presence and absence of added NaCl, implying that the cysteine biosynthetic pathway of each must be partially active, and that addition of NaCl does not inhibit cysteine biosynthesis when mutations in an enzyme other than CysQ is limiting. By contrast, thiosulphate complemented either mutation only in the absence of added NaCl. It is probable that this reflects the site of the secondary mutation being in the sulphate permease, as mutations of the sulphate permease result in decreased ability to transport thiosulphate, and all characterised suppressor mutations of *cysQ* were in the sulphate permease (Neuwald *et al.* 1992).

5. EXPRESSION OF *CYSQ*

The observations of the regulation of the *cysQ-lacZ* fusion strain, 71KL8, in this study implies the involvement of a number of genetic regulatory systems in the control of *cysQ* transcription. The major transcriptional activation appears to occur in response to entry into stationary phase, which has been shown to be at least partially mediated by σ_S. In addition transcription was activated by increased NaCl concentration, although only in rich media in stationary phase. It is possible that this activation is also mediated through σ^S, the transcription of which is also activated in response to increased osmolarity (Hengge-Aronis *et al.* 1991). Stationary phase expression is higher in minimal media than in rich media in both exponential growth and stationary phase, however, the regulation of this response is uncertain.

The results in this study also suggest a tentative link between CAP activation and *cysQ* regulation. Transcriptional activation of *cysQ* was decreased in both exponential and stationary conditions when trehalose was the sole carbon source, conditions in which CAP activation would be occurring. The presence

of a divergently transcribed CAP binding site overlapping the *cysQ* promoter (Liu *et al.* 1986) suggests it to be likely that CAP binding would inhibit *cysQ* transcription. Clarification of this point would be possible by transduction of *cya* or *crp* allleles into 71KL8.

There is also a suggestion of some form of regulation of *cysQ* transcription in response to the CysQ requirements for cysteine biosynthesis. Transcription of *cysQ* was shown to be decreased in response to the presence of *cysQ* in high copy number on plasmid pAD300, in the presence of a secondary mutation (*cysC*) or when reduced sulphur sources, such as cysteine or thiosulphate were supplied. The responsiveness of *cysQ* transcription does not appear to be mediated by the cysteine transactivator CysB, as MM7, a *cysB* derivative of 71KL8, does not show any change in β-galactosidase activity. In addition, Neuwald *et al.* (1992), found no CysB binding sites in the *cysQ* promoter. The lack of CysB regulation would make *cysQ* unique amongst the cysteine biosynthetic genes, as all other genes controlled as part of the cysteine regulon are CysB dependent.

β-galactosidase activities of exponential cultures grown in M9 with various reduced sulphur sources added showed all to have decreased expression compared to M9 alone, with thiosulphate lowest, and with increasing expression levels for methionine and cysteine. These observations suggest two possible explanations: *cysQ* transcription could be directly and inversely responsive to growth rate, with thiosulphate causing the greatest increase in growth rate and thereby the greatest drop in *cysQ* expression levels, a response that might be mediated by σ^S; alternatively, transcription might be directly responsive to the sulphur source, with thiosulphate being the best sulphur source, and thereby resulting in the greatest decrease in transcription. In support of this second hypothesis is the observation that the β-galactosidase activity of M9 + thiosulphate (11.3 Miller units) is little greater than that in LB (6.7 Miller units), whereas expression in M9 alone is 44 Miller units.

The observations in this study did not confirm there to be a direct relationship between increased osmolarity and *cysQ* transcriptional activity in exponential cultures, as observed by McLellan (1992). In this study it was found to be necessary to extensively purify the fusion-bearing strain by subculturing in early exponential growth phase. Without this purification procedure conditions that caused slow growth yielded anomalously high *lacZ* activities because of the

presence of carry-over fusion from the previous stationary phase. This could explain the concentration dependent expression observed by McLellan, as increasing NaCl concentration would cause decreased growth, which would result in slower dilution of fusion protein from the previous stationary phase.

5.1. Aerobic and anaerobic gene expresssion

CysQ is known to be required only during aerobic growth (Neuwald *et al.* 1992). It was found in this study that stationary phase *cysQ* transcription was decreased in anaerobic conditions, but the basis of this regulation could not be ascertained. In *E. coli* there is a system of transcriptional repression of aerobic genes in anaerobic conditions, regulated by ArcA and ArcB proteins (reviewed in Lin and Iuchi 1991). However, it is unknown if this system is active for *cysQ*.

6. CYSQ, THE PRODUCT OF *CYSQ*, IS A PUTATIVE PHOSPHATASE

As already stated CysQ, the *cysQ* gene product, is a putative phosphatase, with homology to sugar specific phosphatases such as IMPase and F-1,6-BPase. A potential role for CysQ function has been suggested by Neuwald *et al.* (1992), who suggest CysQ might be modulating the effects of PAPS, by cleavage of the 3'-phosphoryl group to regenerate APS. In support of this hypothesised function is the considerable homology of the CysQ amino acid sequence to that of IMPase (Neuwald *et al.* 1991). IMPase has far less amino acid homology to F-1,6-BPase than it does to CysQ, yet IMPase and F-1,6-BPase share remarkable similarity in their 3-dimensional protein conformations (Zhang *et al.* 1993). This observation suggests CysQ would also share a similar three dimensional structure. In addition, CysQ has the same 6 conserved aspartate and glutamate residues that are required for the functional site. There are, however, significent differences between the derived amino acid sequences of CysQ and IMPase, differences which are also shared by HAL2. The first aspartate residue of consensus 1 (see figure 5.5) is replaced in CysQ and HAL2 by a serine residue. Also, the arginine residue of consensus 3 is changed in both CysQ and HAL2. This arginine is required by F-1,6-BPase to bind the 6' phosphoryl group, and is suspected to determine the binding specificity of the second phosphoryl group, as well as possibly being involved in dimerisation (Zhang *et al.* 1993). Finally, there are a range of other differences to IMPase

and F-1,6-BPase which are conserved between HAL2 and CysQ. In combination these differences suggest that the putative CysQ phosphatase might have a different second phosphoryl group specificity, which would be required if it's substrate was to be PAPS. Thus, observations of the putative structure of CysQ are consistent with it's hypothesised role.

7. A MODEL FOR CYSQ FUNCTION IN CYSTEINE BIOSYNTHESIS

Observations of the likely enzymatic action of CysQ, and of *cysQ* expression, suggest a dual role for CysQ in cysteine biosynthesis, with different roles during growth in the absence of cysteine, and at stationary phase.

Neuwald *et al.* (1992) suggested three potential roles for CysQ during growth, as a PAPS specific phosphatase, as a helper of CysH enzymatic function, or in sequestration of PAPS. No direct experimental evidence for the mode of action of the *cysQ* gene product was determined in this study, however, observations of the phenotype of *cysQ* mutants confirmed CysQ to be involved in PAPS regulation. In support of this role were the observations that *cysQ* mutations were unstable, with accumulation of suppressor mutations occurring, but only in the absence of a reduced sulphur source and only during growth; stationary phase cultures did not accumulate suppressor mutations. The mutant phenotype was unstable and leaky, with suppressor mutations accumulated that were similar to those accumulated by *cysH* or *trxA, grx* mutants (Gillespie *et al.* 1968, Russel *et al.* 1990). The leakiness of *cysQ* mutants even in the absence of suppressor mutations suggests the cysteine biosynthetic apparatus to be complete, even in the absence of CysQ, providing evidence for CysQ having some modulatory role. In addition the incidence of suppressor mutations at earlier stages in the cysteine biosynthesis pathway suggests an action whereby the secondary mutation down-regulates cysteine biosynthesis, thereby decreasing the requirement for modulation by CysQ, and allowing cysteine biosynthesis to proceed.

During stationary phase CysQ might function as a switch for the sulphur assimilation pathway. This hypothesis requires acceptance of the unproven assumption that CysQ is a phosphatase. A potential model for CysQ function is this: during growth CysQ is active, but only enough to remove any excess PAPS present, preventing PAPS' assumed toxic effects. Upon entry into

stationary phase growth slows, therefore cysteine biosynthesis becomes unnecessary. *CysQ* transcription is turned on via σ_S and the CysQ phosphatase becomes fully active, resulting in all PAPS being degraded to APS. The presence of APS prevents additional sulphate activation occurring, and the sulphate activation pathway becomes blocked.

In support of this hypothesis is the observation that ATP sulphurylase and APS kinase activities decrease at rates greater than expected to be just the result of dilution in 1,2,4-triazole treated cultures (Kredich 1987). Also, both enzymes are sensitive to growth phase and have low to immeassurable activities in late log-early stationary phase cultures. Other cysteine biosynthetic enzymes, such as OAS (thiol)-lyase-A and sulphite reductase, are, however, unaffected by growth phase (Kredich 1987).

8. POSSIBLE ROLES FOR CYSTEINE BIOSYNTHESIS IN OSMOREGULATION

According to Gowrishankar (1985), genes important in osmosensitivity should be either omoresponsive in expression or the gene product should be osmoresponsive in its catalytic function, or they should cause an osmosensitive phenotype if mutated.

In this study there has been no evidence for *cysQ* having a role in osmoregulation. There is, however, evidence that *cysQ* might be important in halotolerance. This linkage does not result from production of osmoprotectants, but rather because the *cysQ* gene product would appear to be inhibited by high salt concentrations. There are two major arguments for this hypothesis.

AM77 is inferred to have partial CysQ activity, as it is able to grow at wild type rates when methionine is supplied, with methionine presumed to act by sparing cysteine. Addition of NaCl to 0.35M, however, prevents supplementation. By contrast K41, which contains a secondary suppressor mutation as well as a *cysQ* $^-$ allele, is supplemented by methionine in both the presence and absence of added NaCl, whereas the growth of WKL10, which is *cysQ* $^-$ but lacks the secondary suppressor mutation, is enhanced only poorly by methionine.

If it is accepted that an at least partially active cysteine biosynthetic pathway is required to enable methionine sparing, then these observations imply that cysteine biosynthesis is partially active in AM77 only in the absence of added NaCl and that cysteine biosynthesis is active in K41 in both the presence and absence of added NaCl. If it is assumed that in AM77 the limitation on cysteine biosynthesis is the partially active CysQ protein, then the absence of growth in the presence of added NaCl suggests the change in salt concentration to have resulted in inhibition of the partially active protein. As methionine restores growth to K41, which also has a limitation in cysteine biosynthesis but at an earlier step, the implication is that the inhibition by added NaCl is specific to the CysQ protein.

In support of this hypothesis, is the observation that supplying reduced sulphur sources to the wild type strain W3110 enhances growth in the presence of osmotic stress. The implication of this observation is that sulphur reduction is one of the rate limiting biosynthetic reactions during growth in minimal conditions in the presence of high salt concentrations.

Consideration of the other members of the IMPase phosphatase family provides support for the hypothesis that CysQ is inhibited by high salt. IMPase, IPPase and F-1,6-BPase are all inhibited noncompetitively by the addition of LiCl, with NaCl also being suggested to inhibit enzyme function (Majerus 1992, Zhang *et al.* 1993). *S. cerevisiae* HAL2 is also suspected to be inhibited by added NaCl. HAL2 was originally isolated as a gene which resulted in increased halotolerance when present in high copy number (Glässer *et al.* 1993). It is likely that HAL2 is a direct homologue of CysQ, as both are required for the sulphur reduction pathway and in addition share amino acid homology different from all other members of the IMPase phosphatase family. While CysQ may be homologous, there are differences in their relative effects; in this study osmotolerance was not enhanced by supplying *cysQ* in high copy. These observations of related proteins would suggest a model whereby salt ions inhibit CysQ by allosteric alterations of the enzyme structure and function.

The observation that the addition of glycine betaine does not restore the growth of AM77, even on M63 + 0.2M NaCl (results not shown), also supports the hypothesis that CysQ is halosensitive. It would be expected that the presence of glycine betaine would be able to compensate for the changes in internal cytoplasmic volume caused by the increased osmolarity. Therefore, the

sensitivity of AM77 at this NaCl concentration implies a sensitivity of the enzyme to NaCl, rather than a change in the internal enviroment of the cell.

To clarify the relationship between *cysQ* and halosensitivity, it would be desirable to examine the effects of other osmolytes on growth. If the activity of CysQ is a limiting factor during growth in increased salt concentrations, then observable differences in growth might be apparent between media in which osmolarity is increased by the addition of NaCl or LiCl, or by another osmolyte such as sucrose. It is possible, however, that the inhibition of CysQ might result from influx of potassium ions, in which case differences in growth may not be apparent. It would also be desirable to check if methionine can complement AM77 in the presence of glycine betaine. If this was so it would be evidence for the inhibition of CysQ being at the level of macromolecular crowding, resulting from a decrease in the cytoplasmic free H_2O wolume (V_F), whereas failure to complement would be evidence for salt ions actively inhibiting the enzyme.

9. FUTURE DIRECTIONS.

This study is just a start in characterising the role of *cysQ* in both stationary and exponential phase. The results in this study, many of which were only obtained shortly before the due date, need further work to really clarify the relationship between the (many) factors examined in this work. Certainly the starting point in future work from my perspective, would be biochemical characterisation of the enzymatic activity of the product of the *cysQ* gene. An initial means of characterising this activity could be to use permeabilised cells. If, as suspected, CysQ is a PAPS specific phosphatase, then PAPS degradation should be able to be detected. The second area in which much work remains to be done, is further characterisation of the genetic regulation of *cysQ* activity. In parallel with this, analysis of the structure of the promoter region of *cysQ* might give some interesting answers as to the nature of genetic regulation of *cysQ*. Finally, study of the possibility of CysQ being halosensitive is also worthy of further work.

REFERENCES

REFERENCES

Altschul, S.F., W. Gish, W. Miller, E.W. Myers, and D.J. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. **215**: 403-410

Arakawa, T., and S.N. Timasheff. 1985. The stabilization of proteins by osmolytes. Biophys. J. **47:** 411-414.

Bachmann, B.J. 1987. Derivatives and genotypes of some mutant derivatives of *Escherichia coli* K12. *In* F.C. Neidhardt, J.L. Ingraham, K.B. Low, B. Magasanik, M. Schaechter, and H.E. Unbarger (ed.), *Escherichia coli* and *Salmonella typhimurium:* cellular and molecular biology. American Society for Microbiology, Washington, D.C.

Bachmann, B.J. 1990. Linkage map of *Escherichia coli* K12, edition 8. Microbiol. Rev. **54:** 130-197.

Birnboim, H.C., and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**: 1513-1523.

Bolivar, F., and K. Backman. 1979. Plasmids of *Escherichia coli* as cloning vectors. Method Enzymol. **68:** 245-280.

Bone, R., J.P. Springer, and J.R. Atack. 1992. Structure of inositol monophosphatase, the putative target of lithium therapy. Proc. Natl. Acad. Sci. USA **89:** 10031-10035.

Boos, W., U. Ehmann, H. Forkl, W. Klein, M. Rimmele, and P. Postma. 1990. Trehalose transport and metabolism in *Escherichia coli*. J. Bacteriol. **172:** 3450-3461.

Booth, I.R., and C.F. Higgins. 1990. Enteric bacteria and osmotic stress: intracellular potassium glutamate as a secondary signal of osmotic stress. FEMS Microbiol. Rev. **75:** 239-246.

Borthakur, D., R.F., Barker, J.W. Latchford, L. Rossen, and A.W.B. Johnston. 1988. Analysis of *pss* genes of *Rhizobium leguminosarum* required for exopolysaccharide synthesis and nodulation of peas: their primary structure and their interaction with *psi* and other nodulation genes. Mol. Gen. Genet. **213:** 155-162.

Botsford, J.L. 1990. Analysis of protein expression in response to osmotic stress in *Escherichia coli*. FEMS Microbiol. Lett. **72:** 355-360.

Brickman, E., L. Soll, and J. Beckwith. 1973. Genetic characterization of mutations which affect catabolite-sensitive operons in *Escherichia coli,* including deletions of the gene for adenyl cyclase. J. Bacteriol. **116:** 582-587.

Cayley, S., B.A. Lewis, H.J. Guttman, and M.T. Record. 1991. Characterization of the cytoplasm of *Escherichia coli*-K-12 as a function of external osmolarity: implications for protein-DNA interactions *in vivo*. J. Mol. Biol. **222:** 281-300.

Cayley, S., B.A. Lewis, and M.T. Record. 1992. Origins of the osmoprotective properties of betaine and proline in *Escherichia coli* K-12. J. Bacteriol. **174:** 1586-1595.

Cayley, S., M.T. Record, and B.A. Lewis. 1989. Accumulation of 3-(N-morpholino)-propanesulfonate by osmotically stressed *Escherichia coli* K-12. J. Bacteriol. **171:** 3597-3602.

Cherest, H., and Y. Surdin-Kerjan. 1992. Genetic analysis of a new mutation conferring cysteine auxotrophy in *Saccharomyces cerevisiae*: updating of the sulfur metabolism pathway. Genetics **130:** 51-58.

Csonka, L.N. 1989. Phsyiological and genetic responses of bacteria to osmotic stress. Microbiol. Rev. **53:** 121-147.

Díaz-Guerra, L., F. Moreno, and J.L. San Millán. 1989. *appR* gene product activates transcription of microcin C7 plasmid genes. J. Bacteriol. **171:** 2906-2908.

Dinnibier, U., E. Limpinsel, R. Schmid, and E.P. Bakker. 1988. Transient accumulation of potassium glutamate and its replacement by trehalose during adaptation of growing cells of *Escherichia coli* K-12 to elevated sodium chloride concentrations. Arch. Microbiol. **150:** 348-357.

Dower, W.J., J.F. Miller, and C.W. Ragsdale. 1988. High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Res. **16:** 6127-6145.

Ebright, R.H. 1993. Transcription activation at class I CAP-dependent promoters. Mol. Microbiol. **8:** 797-802.

Epstein, W. 1986. Osmoregulation by potassium transport in *Escherichia coli.* FEMS Microbiol. Rev. **39:** 73-78.

Eshoo, M.W. 1988. *lac* fusion analysis of the *bet* genes of *Escherichia coli:* regulation by osmolarity, temperature, oxygen, choline and glycine betaine. J. Bacteriol. **170:** 5208-5215.

Fabiny, J.M., A. Jayakumar, A.C. Chinault, and E.M. Barnes. 1991. Ammonium transport in *Escherichia coli:* localization and nucleotide sequence of the *amtA* gene. J. Gen. Microbiol. **137:** 983-989.

Ganzhorn, A.J., P. Vincendon, and J.T. Pelton. 1993. Structural characterization of *myo*-inositol monophosphatase from bovine brain by secondary structure prediction, fluorescence, circular dichroism and Raman spectography. Biochim. Biophys. Acta. **1161:** 303-310.

Geever, R.F., L. Huiet, J.A. Baum, B.M. Tyler, V.B. Patel, B.J. Rutledge, M.E. Case and N.H. Giles. 1989. DNA sequence, organization and regulation of the *qa* gene cluster of *Neurospora crassa.* J. Mol. Biol. **207:** 15-34.

Gillespie, D., M. Demerec, and H. Itikawa. 1968. Appearance of double mutants in aged cultures of *Salmonella typhimurium* cysteine-requiring strains. Genetics **59:** 433-442.

Gläser, H.U., D. Thomas, R. Gaxiola, F. Montrichard,Y. Surdinkerjan, and R. Serrano. 1993. Salt tolerance and methionine biosynthesis in *Saccharomyces cerevisiae* involve a putative phosphatase gene. EMBO Journal. **12:** 3105-3110.

Glæver, H.M., O.B. Styrvold, I. Kaasen, and A.R. Strøm. 1988. Biochemical and genetic characterization of osmoregulatory trehalose synthesis in *Escherichia coli.* J. Bacteriol. **170:** 2841-2949.

Gowrishankar, J. 1985. Identification of Osmoresponsive genes in *Escherichia coli*: evidence for participation of potassium and proline transport systems in osmoregulation. J. Bacteriol. **164:** 434-445.

Gutierrez, C., M. Ardourel, E. Bremer, A. Middendorf, W. Boos, and U. Ehmann. 1989. Analysis and DNA sequence of the osmoregulated *treA* gene encoding the periplasmic trehalase of *Escherichia coli* K12. Mol. Gen. Genet. **217:** 347-354.

Hallenbeck, P.C., M.A. Clark, and E.L. Barrett. 1989. Chracterization of anaerobic sulfite reduction by *Salmonella typhimurium* and the purification of the anaerobically induced sulfite reductase. J. Bacteriol. **171:** 3008-3015.

Harris, C.L. 1981. Cysteine and growth inhibition of *Escherichia coli:* threonine deaminase as the target enzyme. J. Bacteriol. **145:** 1031-35.

Hawkins, A.R., H.K. Lamb, M. Smith, J.W. Keyte, and C.F. Roberts. 1988. Molecular organisation of the quinic acid utilization (*QUT*) gene cluster in *Aspergillus nidulans*. Mol. Gen. Genet. **214:** 224-231.

Hellinga and Evans. 1985. Nucleotide sequence and high-level expression of the major *Escherichia coli* phosphofructokinase. Eur. J. Biochem. **149**: 363-373.

Hendrickson,W., and K.E. Rudd. 1991. J. Bacteriol. **174**: 3836-3837.

Hengge-Aronis, R., W. Klein, R. Lange, M. Rimmele, and W. Booth. 1991. Trehalose synthesis genes are controlled by the putative sigma factor encoded by *rpoS* and are involved in stationary-phase thermotolerance in *Escherichia coli*. J. Bacteriol. **173:** 7918-7924

Hengge-Aronis, R. 1993. Survival of hunger and stress: the role of *rpoS* in early stationary phase gene regulation in *E. coli*. Cell **72:** 165-168.

Hengge-Aronis, R. The role of *rpoS* in early stationary phase gene regulation in *Escherichia coli* K12. *In* S. Kjelleberg (ed.), Starvation in bacteria, in press. Plenum Press, New York.

Hengge-Aronis, R., and D. Fischer. 1992. Identification and molecular analysis of *glgS*, a novel growth-phase-regulated and *rpoS*-dependent gene involved in glycogen synthesis in *Escherichia coli*. Mol. Microbiol. **6:** 1877-1886.

Hengge-Aronis, R., R. Lange, N. Henneberg, and D. Fischer. 1993. Osmotic regulation of *rpoS*-dependent genes in *Escherichia coli*. J. Bacteriol. **175:** 259-265.

Hernández-Chico, C., J.L. San Millán, R. Kolter, and F. Moreno. 1986. Growth phase and *ompR* regulation of transcription of microcin B17 genes. J. Bacteriol. **167:** 1058-1065.

Holmes, D.S., and M. Quigley. (1981). Anal. Biochem. **114:** 193-197.

Hryniewicz, M.M., and N.M. Kredich. 1991. The *cysP* promoter of *Salmonella typhimurium*: characterization of two binding sites for CysB protein, studies of in vivo transcription initiation, and demonstration of the anti-inducer effects of thiosulfate. J. Bacteriol. **173:** 5876-5886.

Hryniewicz, M., A. Sirko, A. Palucha, A. Böck, and D. Hulanicka. 1990. Sulfate and thiosulfate transport in *Escherichia coli* K12:

identification of a gene encoding a novel protein involved in thiosulfate binding. J. Bacteriol. **172:** 3358-3366.

Huang, C.J., and E.L. Barrett. 1990. Identification and cloning of genes involved in anaerobic sulfite reduction by *Salmonella typhimurium*. J. Bacteriol. **172:** 410-4102.

Ingraham, J. 1987. Effect of temperature, pH, water activity, and pressure on growth, p.1543-1554. *In* F.C. Neidhardt, J.L. Ingraham, K.B. Low, B. Magasanik, M. Schaechter, and H.E. Unbarger (ed.), *Escherichia coli* and *Salmonella typhimurium:* cellular and molecular biology. American Society for Microbiology, Washington, D.C.

Innis, M.A., D.H. Gelfand, and J.J. Sninsky (ed). 1990. PCR protocols. Academic Press Inc.

Jayakumar, A., S.J. Hwang, J.M.Fabiny, E.M. Barnes, and A.C. Chinault. 1989. Isolation of an ammonium or methylammonium ion transport mutant of *Escherichia coli* and complementation by the cloned gene. J. Bacteriol. **171:** 996-1001.

Jayakumar, A., K.E. Rudd, J.M. Fabiny, and E.M. Barnes. 1991. Localization of the *Escherichia coli amtA* gene to 95.8 minutes. J. Bacteriol. **173:** 1572-1573.

Jebbar, M., R. Talibart, K. Gloux, T. Bernard, and C. Blanco. 1992. Osmoprotection of *Escherichia coli* by ectoine: uptake and accumulation characteristics. J. Bacteriol. **174:** 5027-5035.

Kaasen, I., P. Falkenberg, O.B. Styrvold, and A.R. Strøm. 1992. Molecular cloning and physical mapping of the *otsBA* genes, which encode the osmoregulatory trehalose pathway of *Escherichia coli:* evidence that transcription is activated by KatF (AppR). J. Bacteriol. **174:** 889-898.

Kleckner, N., J. Bender, and S. Gottesman. 1991. Uses of transposons with emphasis on Tn*10*. Methods Enzymol. **204:** 139-180.

Koch, A.L. 1982. On the growth and form of *Escherichia coli*. J. Gen. Microbiol. **128:** 2527-2540.

Kohara, Y., K. Akiyama, and K. Isono. 1987. The physical map of the whole *E. coli* chromosome: application of a new strategy for rapid analysis and sorting of a large genomic library. Cell **50:** 495-508.

Kredich, N.M. 1987. Biosynthesis of cysteine, p.419-428. *In* F.C. Neidhardt, J.L. Ingraham, K.B. Low, B. Magasanik, M. Schaechter, and H.E. Unbarger (ed.), *Escherichia coli* and *Salmonella typhimurium:* cellular and molecular biology. American Society for Microbiology, Washington, D.C.

Kredich, N.M. 1992. The molecular basis for positive regulation of *cys* promoters in *Salmonella typhimurium* and *Escherichia coli*. Mol. Microbiol. **6:** 2747-2753.

Kren, B., D. Parsell, and J.A.Fuchs. 1988. Isolation and characterization of an *Escherichia coli* K-12 mutant deficient in glutaredoxin. J.Bacteriol. **170:** 308-315.

Krone, F.A., G. Westphal, H.E. Meyer, and J.D. Schwenn. 1990. PAPS-reductase of *Escherichia coli* - correlating the N-terminal amino-acid-sequence with the DNA of Gene *cysH*. FEBS Letters. **260:** 6-9.

Krone, F.A., G. Westphal, and J.D. Schwenn. 1991. Characterization of the gene *cysH* and of its product phospho-adenylylsulphate reductase from *Escherichia coli*. Mol. Gen. Genet. **225:** 314-319.

Kulakauskas, S., P.M. Wikström, and D.E. Berg. 1991. Efficient introduction of cloned mutant alleles into the *Escherichia coli* chromosome. J. Bacteriol. **173:** 2633-2638.

Lange, R., and R. Hengge-Aronis. 1991. Growth phase-regulated expression of *bolA* and morphology of stationary-phase *Escherichia coli* cells are controlled by the novel sigma factor σ^S. J. Bacteriol. **173:** 4474-4481.

Lange, R., and R. Hengge-Aronis. 1991. Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. Mol. Microbiol. **5:** 49-59.

Larsen, P.I., L.K. Sydnes, B. Landfald, and A.R. Strøm. 1987. Osmoregulation in *Escherichia coli* by accumulation of organic osmolytes: betaines, glutamic acid, and trehalose. Arch. Microbiol. **147:** 1-7.

Leyh, T.S., T.F. Vogt, and Y. Suo. 1992. The DNA sequence of the sulfate activation locus from *Escherichia coli* K12. J. Biol. Chem. **267:** 10405-10410.

Lin, E.C.C., and S. Iuchi. 1991. Regulation of gene expression in fermentative and respiratory systems in *Escherichia coli* and related bacteria. Annu. Rev. Genet. **25:** 361-87.

Liu, J., and I.R. Beacham. 1990. Transcription and regulation of the *cpdB* gene in *Escherichia coli* K12 and *Salmonella typhimurium* LT2: evidence for modulation of constitutive promoters by cyclic AMP-CRP complex. Mol. Gen. Genet. **222:** 161-165.

Loewen, P. C., and B.L. Triggs. 1984. Genetic mapping of *katF*, a locus that with *katE* affects the synthesis of a second catalase species in *Escherichia coli*. J. Bacteriol. **160:** 668-675.

Loewen, P.C., I. von Ossowski, J. Switala, and M.R. Mulvey. 1993. KatF (σ^S) synthesis in *Escherichia coli* is subject to posttranscriptional regulation. J. Bacteriol. **175:** 2150-2153.

Lopalito, J., S. Bortner, and J. Beckwith. 1986. Mutations in a new chromosomal gene of *Escherichia coli* K-12, *pcnB*, reduce plasmid copy number of pBR322 and its derivatives. Mol. Gen. Genet. **205:** 285-309.

Majerus, P.W. 1992. Inositol phosphate biochemistry. Annu. Rev. Biochem. **61:** 225-250.

Mark, D.F., J.W. Chase, and C.C. Richardson. 1977. Genetic mapping of *trxA*, a gene affecting thioredoxin in *Escherichia coli* K-12. Mol. Gen. Genet. **155**: 145-152

Matin, A. 1991. The molecular basis of carbon-starvation-induced general resistance in *Escherichia coli*. Mol. Microbiol. **5:** 3-10.

Matin, A., E.A. Auger, P.H. Blum, and J.E. Schultz. 1989. Genetic basis of starvation survival in nondifferentiating bacteria. Annu. Rev. Microbiol. **43:** 293-316.

McAllister, G., P. Whiting, E.A. Hammond, M.R. Knowles, J.R. Atack, F.R. Bailey, R. Maigetter, and C.I. Ragan. 1992. cDNA cloning of human and rat brain *myo*-inositol monophosphatase. Biochem. J. **284:** 749-754.

McCann, M.P., C.D. Fraley, and A. Matin. 1993. The putative σ factor KatF is regulated posttranscriptionally during carbon starvation. J. Bacteriol. **175:** 2143-2149.

McCann, M.P., J.P. Kidwell, and A. Matin. 1991. The Putative σ factor KatF has a central role in development of starvation-mediated general resistance in *Escherichia coli*. J. Bacteriol. **173:** 4188-4194.

McLaggan, D., T.M. Logan, D.G. Lynn, and W. Epstein. 1990. Involvement of γ-glutamyl peptides in osmoadaptation of *Escherichia coli*. J. Bacteriol. **172:** 3631-3636.

McLaggan, D., and W. Epstein. 1991. *Escherichia coli* accumulates the eukaryotic osmolyte taurine at high osmolarity. FEMS Microbiol. Lett. **81:** 209-214.

McClellan, A. 1992. Identification and cloning of *otsC*, an osmoresponsive gene required for trehalose synthesis during osmoadaptation in *Escherichia coli*. Master of Science thesis. University of Canterbury.

Miller, J.H. 1972. Experiments in molecular genetics. Cold Spring Harbour Laboratory, Cold Spring Harbour, New York.

Mulvey, M.R., and P.C. Loewen. 1989. Nucleotide sequence of *katF* of *Escherichia coli* suggests KatF protein is a novel σ transcription factor. Nucleic Acids Res. **17:** 9979-9991.

Nagai, H., H. Yuzawa, and T. Yura. 1991. Interplay of two cis-acting mRNA regions in translational control of synthesis during the heat shock response of *Escherichia coli*. Proc. Natl. Acad. Sci. USA. **88:** 10515-10519.

Neuwald, A.F., B.R. Krishnan, I. Brikun, S. Kulakauskas, K. Suziedelis, T. Tomcsanyi, T.S. Leyh, and D.E. Berg. 1992. *cysQ*, a gene needed for cysteine synthesis in *Escherichia coli* K-12 only during aerobic growth. J. Bacteriol. **174:** 415-425.

Neuwald, A.F., J.D. York, and P.W. Majerus. 1991. Diverse proteins homologous to inositol monophosphatase. FEBS Lett. **294:** 16-18.

Ní Bhriain, N., C.J. Dorman, and C.F. Higgins. 1989. An overlap between osmotic and anaerobic stress responses: a potential role for DNA supercoiling in the coordinate regulation of gene expression. Mol. Microbiol. **3:** 933-942.

Norris, J.R., and D.W. Ribbons. (ed). 1971. Methods in Microbiology, vol 5b. Academic Press inc., London.

O'Byrne, C.P., N. Ní Bhriain, and C.J. Dorman. 1992. The DNA supercoiling-sensitive expression of the *Salmonella typhimurium his* operon requires the *his* attenuator and is modulated by anaerobiosis and by osmolarity. Mol. Microbiol. **6:** 2467-2476.

Ostrowski, J., and N.M. Kredich. 1990. In vitro interactions of CysB protein with the *cysJIH* promoter of *Salmonella typhimurium:* inhibitory effects of sulfide. J. Bacteriol. **172:** 779-785.

Ostrowski, J., L.M. Siegel, B.E. Miller, J.Y. Wu, D.C. Rueger, and N.M. Kredich. 1989. Characterization of the *cysJIH* regions of *Salmonella typhimurium* and *Escherichiacoli*-B DNA sequences of *cysI* and *cysH* and a model for the siroheme-Fe_4S_4 active center of sulfite reductase

hemoprotein based on amino-acid homology with spinach nitrite reductase. J. Biol. Chem. **264:** 5726-5737.

Pissowotzki, K., K. Mansouri, and W. Piepersberg. 1991. Genetics of streptomycin production in *Streptomyces griseus:* molecular structure and putative function of genes *strELMB2N*. Mol. Gen. Genet. **231:** 113-123.

Postgate, J.R. 1963. The examination of sulphur auxotrophs: a warning. J. Gen. Microbiol. **30:** 481-484.

Repoila, F., and C. Gutierrez. 1991. Osmotic induction of the periplasmic trehalase in *Escherichia coli* K12: characterization of the *treA* gene promoter. Mol. Microbiol. **5:** 747-755.

Rod, M.L., K.Y. Alam, P.R. Cunningham, and D.P. Clark. 1988. Accumulation of trehalose by *Escherichia coli* K-12 at high osmotic pressure depends on the presence of amber suppressors. J. Bacteriol. **170:** 3601-3610.

Russel, M., P. Model, and A. Holmgren. 1990. Thioredoxin or glutaredoxin in *Escherichia coli* is essential for sulfate reduction but not for deoxyribonucleotide synthesis. J. Bacteriol. **172:** 1923-1929.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. 2nd ed. 3 vols. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Schwenn, J.D., F.A. Krone, and K. Nusmann. 1988. Yeast PAPS reductase: properties and requirements of the purified enzyme. Arch. Microbiol. **150:** 313-319.

Siegele, D.A., and R. Kolter. 1992. Life after log. J. Bacteriol. **174:** 345-348.

Sirko, A., M. Hryniewicz, D. Hulanicka, and A. Böck. 1990. Sulfate and thiosulfate transport in *Escherichia coli* K-12: nucleotide sequence and expression of the *cysTWAM* gene cluster. J. Bacteriol. **172:** 3351-3357.

Smith, M.W., and F.C. Neidhart. 1983. Proteins induced by anaerobiosis in *Escherichia coli.* J. Bacteriol. **154:**336-343.

Sørensen, M.A., and S. Pedersen. 1991. Cysteine, even in low concentrations, induces transient amino acid starvation in *Escherichia coli.* J. Bacteriol. **173:** 5244-5246.

Spector, M.P., and C.L. Cubitt. 1992. Starvation inducible loci of *Salmonella typhimurium:* regulation and roles in starvation survival. Mol. microbiol. **6:** 1467-1476.

Strøm, A.R., and I. Kaasen. 1993. Trehalose metabolism in *Escherichia coli:* stress protection and stress regulation of gene expression. Mol. Microbiol. **8:** 205-210.

Styrvold, O.B., and A.R. Strøm. 1991. Synthesis, accumulation, and excretion of trehalose in osmotically stressed *Escherichia coli* K-12 strains: influence of amber suppressors and function of the periplasmic trehalase. J. Bacteriol. **173:** 1187-1192.

Tanaka, K., Y. Takayanagi, N. Fujita, A. Ishihama, and H. Takahashi. 1993. Heterogeneity of the principal σ factor in *Escherichia coli:* the *rpoS* gene product, σ^{38}, is a second principal σ factor of RNA polymerase in stationary-phase *Escherichia coli.* Proc. Natl. Acad. Sci. USA **90:** 3511-3515.

Tei, H., K. Murata, and A. Kimura. 1990. Molecular cloning of the *cys* genes (*cysC, cysD, cysH, cysI, cysJ,* and *cysG*) responsible for cysteine biosynthesis in *Escherichia coli* K-12. Biotech. App. Biochem. **12:** 212-216.

Tei, H., K. Murata, and A. Kimura. 1990. Structure and expression of *cysX*, the second gene in the *Escherichia coli* K-12 *cysE* Locus. Biochem. Biophys. Res. Commun. **167**: 948-955.

Tormo, A., M. Almirón, and R. Kolter. 1990. *surA*, an *Escherichia coli* gene essential for survival in stationary phase. J. Bacteriol. **172:** 4339-4347.

Uria-Nickelsen, M.R., E.R. Leadbetter, and W. Godchaux. 1993. Sulphonate utilization by enteric bacteria. J. Gen. Microbiol. **139:** 203-208.

Volkert, M.R., L.I. Hajec and D.C.Nguyen. 1989. Induction of the alkylation-inducible *aidB* gene of *Escherichia coli* by anaerobiosis. **171:** 1196-1198

Wang, J.-Y., and M. Syvanen. 1992. DNA twist as a transcriptional sensor for environmental changes. Mol. Microbiol. **6:** 1861-1866.

Way, J.C., M.A. Davis, D. Morisato, D.E. Roberts, and N. Kleckner. 1984. New Tn*10* derivatives for transposon mutagenesis and for construction of *lacZ* operon fusions by transposition. Gene **32:** 369-379.

Welsh, D.T., R.H. Reed, and R.A. Herbert. 1991. The role of trehalose in the osmoadaptation of *Escherichia coli* NCIB 9484: interaction of trehalose, K^+ and glutamate during osmoadaptation in continuous culture. J. Gen. Microbiol. **137:** 745-750.

Yancy, P.H., M.E. Clark, S.C. Hand, R.D. Bowlus, and G.N. Somero. 1982. Living with water stress: evolution of osmolyte systems. Science **217:** 1214-1222.

Yanisch-Perron, C., J. Viera, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequence of M13mp18 and pUC19 vectors. Gene **33:** 103-119.

Yano, R., H. Nagai, K. Shiba, and T. Yura. 1990. A mutation that enhances synthesis of σ^{32} and suppresses temperature-sensitive growth of the *rpoH15* mutant of *Escherichia coli*. J. Bacteriol. **172:** 2124-2130.

Yelton, D.B., and S.L. Peng. 1989. Identification and nucleotide sequence of the *Leptospira biflexa* serovar *patoc trpE* and *trpG* Genes. J. Bacteriol. **171:** 2083-2089.

Zhang, Y., J.Y. Liang, and W.N. Lipscomb. 1993. Structural similarities between fructose-1, 6-bisphosphatase, and inositol monophosphatase. Biochem. Biophys. Res. Commun. **190:** 1080-1083.

APPENDICES

APPENDIX I

SUPPLEMENTS

0.5M IPTG

11.9g IPTG
dH_2O to 100ml

30mg/ml X-Gal

1.5g X-Gal
dimethylformamide to 50 ml

1M glycine betaine

11.7g glycine betaine
dH_2O to 100ml

1M choline

10.4g choline
dH_2O to 100ml

Amino acids

All amino acids were dissolved in dH_2O to 100x their working concentrations and then filter sterilised.

BUFFERS AND SOLUTIONS

STET buffer (Holmes and Quigley 1981)

8%	sucrose
5%	triton X-100
50mM	EDTA
50mM	tris-HCl (pH8.0)

dH_2O to 100ml, autoclave and store at room temperature.

5 x *Bal31* buffer (Sambrook *et al.* 1989)

3 M	NaCl
60 mM	$CaCl_2$
60mM	$MgCl_2$
100mM	tris-HCl (pH8.0)
1mM	EDTA (pH 8.0)

10 x PCR buffer (Innis *et al.* 1990)

200mM	tris-HCl pH 8.3
250mM	KCl
15mM	$MgCl_2$
0.5%	tween 20
1000µg/ml	sterile BSA

1x Taq polymerase dilution buffer (Pharmacia recipe)

50mM	tris-HCl pH7.6
100mM	NaCl
0.1mM	EDTA
1mM	DTT
50%	glycerol

filter sterilise

End filling buffer (Sambrook *et al.* 1989)

500mM	tris-HCl
100mM	$MgCl_2$
10 mM	DTT
500µg	BSA

3M sodium acetate (pH4.8)

24.6g	sodium acetate

dH_2O to 100ml
adjust pH to 4.8

TES

10mM	tris-HCl (pH 8.0)
1mM	EDTA
100mM	NaCl

TE

10mM	tris-HCl pH 8.0
1mM	EDTA pH 8.0

50x TAE

242g	trizma Base
2M	tris-acetate
100ml	0.5M EDTA pH 8.0
57.1ml	glacial acetic acid

dH_2O to 1 litre pH 8.0.

10x TBE

121.1g	trizma base
51.35g	boric acid
3.72g	EDTA

dH_2O to 1 litre pH 8.0.

10% SDS

10g	SDS

dH_2O to 100ml.

0.5M EDTA

18.612g	EDTA

dH_2O to 100ml pH 8.0.

Loading buffer for agarose gels

30%	glycerol
0.25%	Bromophenol blue
0.25%	Xylene cyanol

RNaseA to a final concentration of 10ug/ml was added when required.

200mM EGTA

7.6g	EGTA

dH_2O to 100ml

dNTP solution (for PCR and end filling)

Equal volumes of 20mM stocks of dATP, dCTP, dGTP and dTTP were mixed together

Alkaline lysis of DNA

Solution I

50mM	glucose
10mM	EDTA
25mM	tris HCl pH 8.0
2mg/ml	lysozyme

Solution II

1%	SDS
0.2M	NaOH

Solution III

3M	sodium acetate pH 4.8

LiCl plasmid preparation

Solution I

1%	glucose
25mM	tris-HCl pH 8.0
10mM	EDTA

Solution II

0.2%	NaOH
1%	SDS

Solution III

5M	potassium acetate

5M LiCl

21.2g	LiCl
dH_2O to 100ml	

2.5M NaCl- 20% PEG

14.6g	NaCl
20g	PEG 7000-9000
dH_2O to 100ml	

TRANSFORMATION AND TRANSDUCTION

$CaCl_2$ method

Solution I

10mM NaCl

Solution II

100mM $CaCl_2$

10% glycerol

10ml glycerol
90ml dH_2O
filter sterilise

0.1M citrate buffer (Miller 1972)

235ml 0.1M citric acid
765ml 0.1M sodium citrate

β-GALACTOSIDASE DETERMINATIONS (Miller 1972)

Z buffer

16.1g $Na_2HPO_4.7H_2O$
5.5g $NaH_2PO_4.H_2O$
0.75g KCl
0.24g $MgSO_4.7H_2O$
2.7ml β-mercaptoethanol
dH_2O to 1000ml
adjust pH to 7.0

4mg/ml ONPG

0.4g ONPG
dH_2O to 100ml

0.1M phosphate buffer (pH 7.0)

61.0ml 1M Na_2HPO_4
39.0ml 1M NaH_2PO_4

1M Na_2CO_3

10.6g Na_2CO_3
dH_2O to 100ml

SEQUENCING

Annealing buffer

280mM tris-HCl pH7.5
100mM $MgCl_2$
350mM DTT

Labelling mix

2mM dCTP
2mM dGTP
2mM dTTP

Termination mixes

10mM $MgCl_2$
40mM tris-HCl pH7.5
50mM NaCl
150mM dATP, dCTP, dGTP, dTTP

A includes ddATP
C includes ddCTP
G includes ddGTP
T includes ddTTP

Stop solution

20mM	EDTA pH7.5
0.05%	xylene cyanol
0.05%	bromophenol blue
95%	deionised formamide

6% polyacrylamide gel

42g	urea
14.5ml	38%/2% polyacrylamide stock solution
10ml	TBE

dH_2O to 99.2ml and then add

800µl	10% ammonium persulphate
80µl	TEMED

Preparation of electrophoresis plates

5ml repel silane was applied to the clean backplate (twice). It was then rinsed with dH_2O

The clean front plate was immersed for 1 hour in bind silane solution (8ml bind silane, 2000ml dH_2O)

10% ammonium persulphate

0.5g	ammonium persulphate
dH_2O to 5ml	

38%/2% polyacrylamide stock solution

38g	acrylamide
2g	bis-acrylamide
dH_2O to 100ml	

APPENDIX II

MEDIA

All media

For all media 15g Agar was added per litre for plates.

M9 (Miller 1972)

per litre

6g	Na_2HPO_4
3g	KH_2PO_4
0.5g	NaCl
1g	NH_4Cl

dH_2O to 1 litre
and, after autoclaving:

10ml	0.01M $CaCl_2$
10ml	20% glucose (or alternative carbon source)
1ml	20% $MgSO_4.7H_2O$
500µl	1% thiamine

LBMM

per litre

10g	bactotryptone
5g	bactoyeast
5g	NaCl

dH_2O to 1 litre
and, after autoclaving:

10ml	20% maltose
12.5ml	20% $MgSO_4$
100µl	1% thiamine

Luria-Bertani medium (LB)

10g	bactotryptone
5g	bactoyeast extract
5g	NaCl

dH_2O to 1 litre and autoclave

M63 Media

13.6g	KH_2PO_4
2g	$(NH_4)_2SO_4$
0.5mg	$FeSO_4.7H_2O$

dH_2O to 1 litre and autoclave.

After autoclaving add

1ml	20% $MgSO_4$
0.5ml	1% thiamine
10ml	20% glucose

H-top agar

10g	bactotryptone
8g	NaCl
8g	agar

dH_2O to 1 litre.

SOC Media (for electroporation)

2g	bactotryptone
0.5g	bactoyeast extract
200ml	5M NaCl
250ml	1M KCl
1ml	1M $MgCl_2$
10ml	0.1M $MgSO_4$
2ml	1M glucose

dH_2O to 100 ml.

Tryptone broth (TB)

10g	bactotryptone
8g	NaCl

dH_2O to 1 litre.

APPENDIX III

AMINO ACID CONCENTRATIONS

Amino acid concentrations

amino acid	single letter codes	working conc. (mM)
Alanine	A	0.47
Arginine	R	0.6
Asparagine	N	0.32
Aspartate	D	0.3
Cysteine	C	0.3
Glutamate	E	5.0
Glutamine	Q	5.0
Glycine	G	0.13
Histidine	H	0.1
Isoleucine	I	0.3
Leucine	L	0.3
Lysine	K	0.3
Methionine	M	0.3
Phenylalanine	F	0.3
Proline	P	2.0
Serine	S	4.0
Threonine	T	0.3
Tryptophan	W	0.1
Tyrosine	Y	0.1
Valine	V	0.3

APPENDIX IV

ABBREVIATIONS

The following abbreviations were used in this thesis

dH20	glass double distilled H_2O
dATP	deoxy adenosine triphosphate
dCTP	deoxy cytosine triphosphate
dGTP	deoxy guanosine triphosphate
dNTP	deoxyribonucleotide triphosphate
dTTP	deoxy thymidine triphosphate
rpm	revolutions per minute
DNA	deoxyribonucleic acid
M	molar
mM	millimolar
EGTA	ethylene glycol bis-N,N,N',N'-tetra acetic acid
PEG	poly ethylene glycol
RNase	ribonuclease
SDS	sodium dodecyl sulphate
U	units of enzyme
PCR	polymerase chain reaction
Amt	ammonium (methylammonium) transport
APS	adenosine 5'-phosphosulphate
PAPS	3'-phosphoadenosine 5'-phosphosulphate
cAMP	cyclic adenosine mono phosphate
TEMED	N,N,N',N'-tetra methyl ethylene diamine
EDTA	di sodium ethylene diamine tetra acetate
DTT	di thio threitol
ONPG	*o*-nitrophenyl-β-D-galactoside
kb	kilo base pairs
bp	base pairs
kD	kilo Daltons
ORF	open reading frame
mRNA	messenger RNA
IMP	inositol monophosphatase
OAS	*O*-acetyl serine
MMS	methyl methanesulphonate

APPENDIX V

PLASMID MAPS

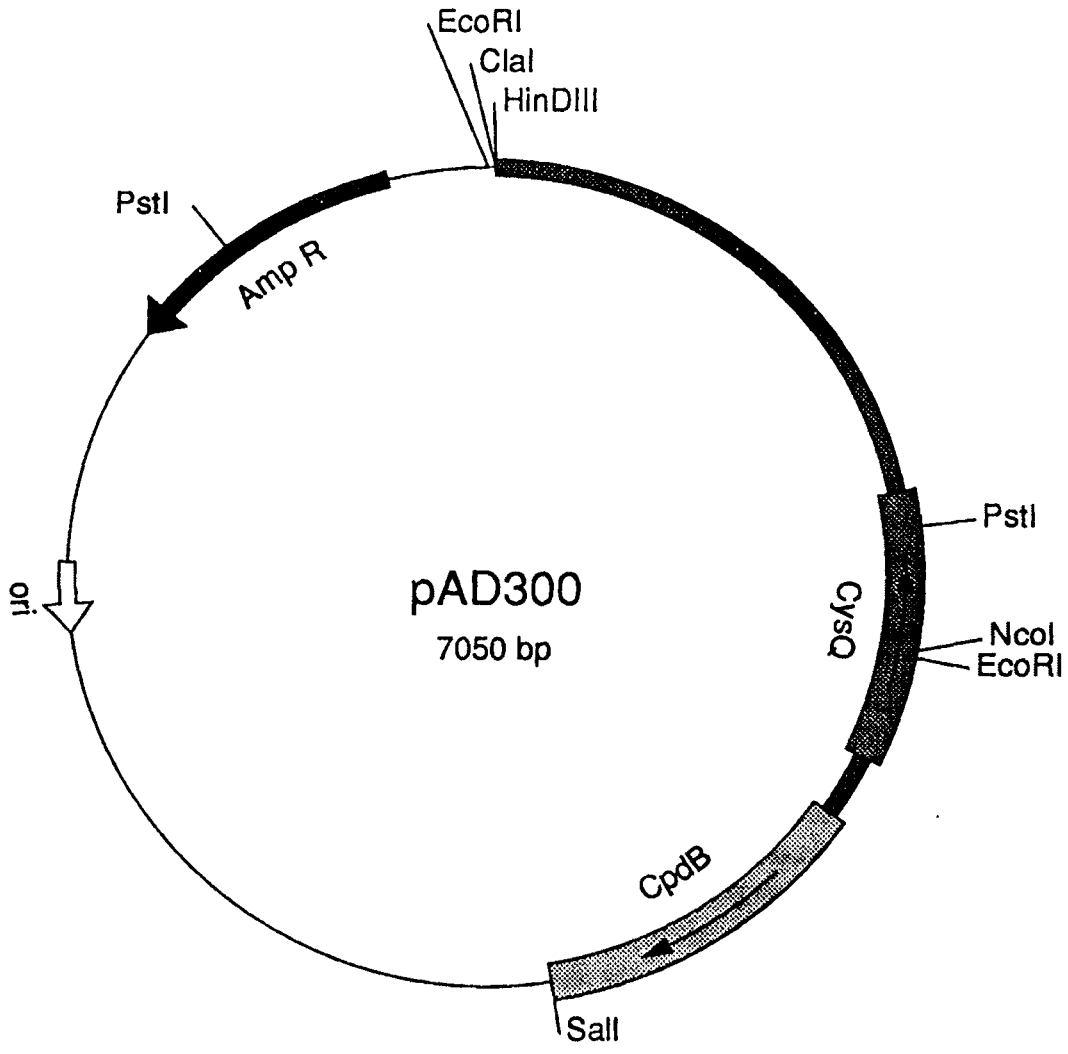


Figure 5.1. pAD300 contains a 3.2 kb *SalI-HinDIII* fragment of chromosomal DNA from λ K656 (medium cross-hatched fragment) cloned into pBR322 (thin fragment) (McLellan 1992).

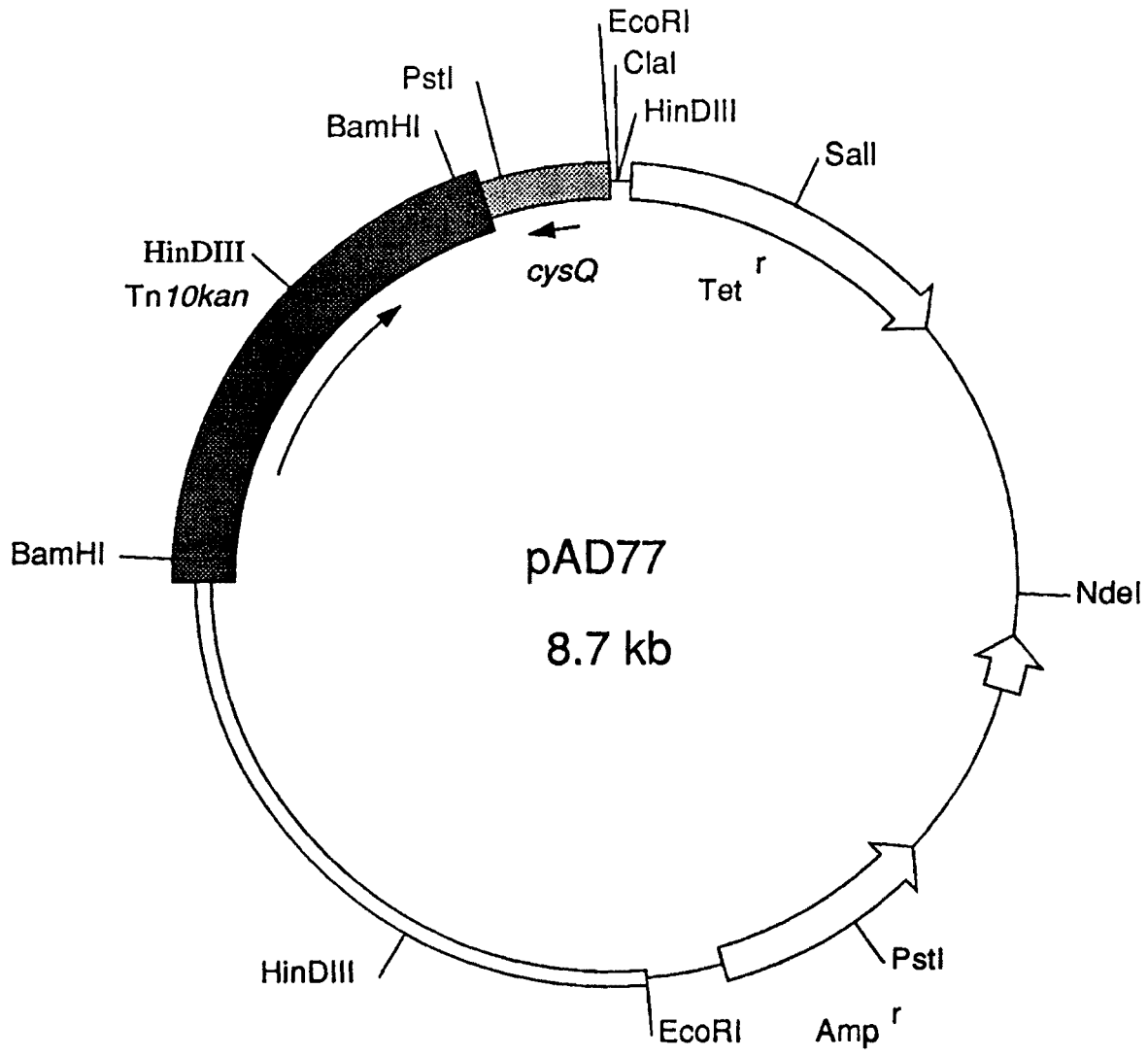


Figure 5.2. pAD77 contains the cloned mini Tn*10kan* insertion from AM77. An *EcoRI* fragment from AM77 is cloned into pBR322. The direction of transcription of the Kan^r element is opposite to that of *cysQ*, and towards the *cysQ* promoter (McLellan 1992).

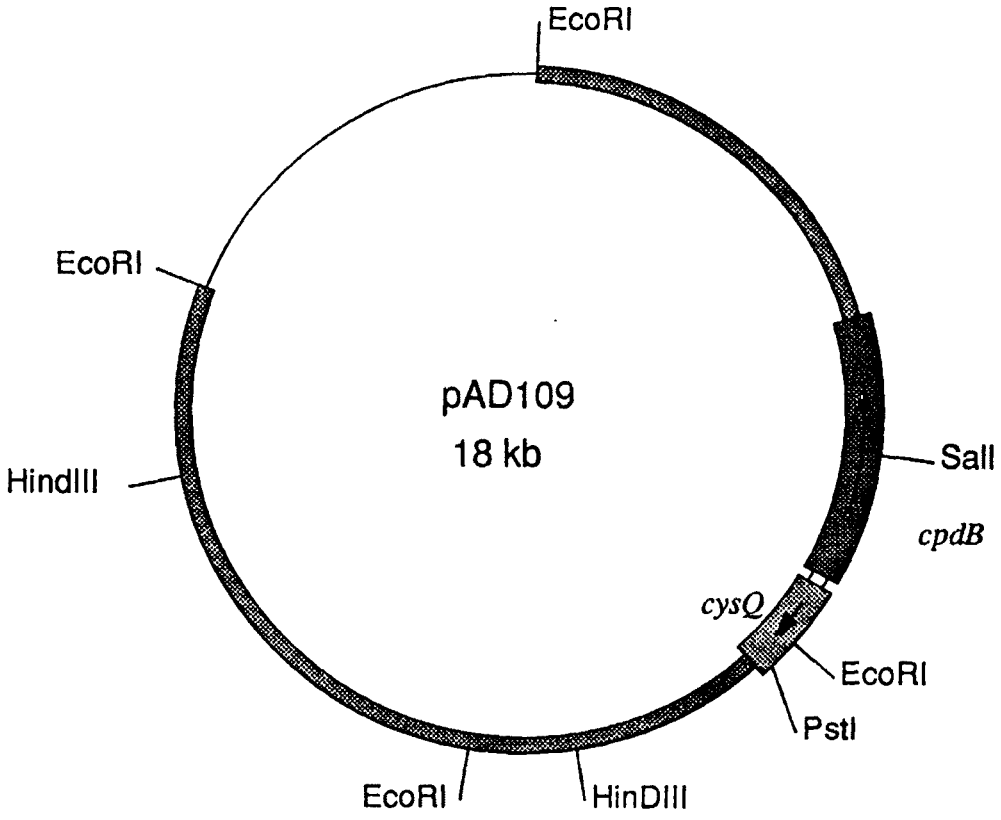


Figure 5.3. pAD109 contains the complete 13 kb *EcoRI* fragment of genomic DNA from λ K656 cloned into the low copy plasmid pJEL109 (McLellan 1992).

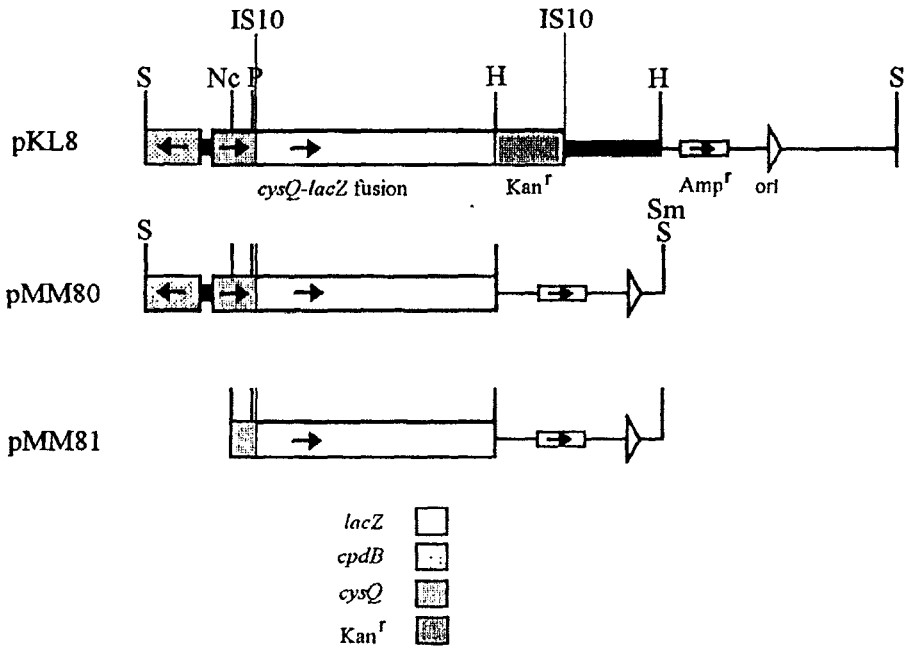


Figure 5.4. Subcloning of pMM80, pMM81, pMM100 and pMM101. pKL8 is a derivative of pAD300, containing a mini Tn*10-LK* fusion to *cysQ*. All pMM derivatives are pUC18 based. pMM80 was constructed by subcloning of a *SalI-HinDIII* fragment from pKL8, while pMM81 was a *SmaI-NcoI* cut self ligated deletion of pMM80. pKL10, pMM100 and pMM101, are identical to pKL8 and derivatives, except that the mini Tn*10-LK* insertion is 101 bp closer to the *cysQ* promoter, resulting in loss of the *PstI* site of *cysQ*. The mini Tn*10-LK* insertion is the region bounded within the IS10 sequences. Restriction enzyme site abbreviations are: H = *HinDIII*, Nc = *NcoI*, P = *PstI*, S = *SalI*. Vectors are shown as thin lines.

```
MADP--WQECMDYAVTLARQAGEVVCEAIKNEMNVMLKSSPVDLVTATDQKVEKMLISSI   58 HuIMP
M-----HPMLNIAVRAARKAGNLIAKNYETPDAVEASQKGSNDFVTNVDKAAEAVIIDTI   55 SuhB
M-LDQVC-------QLARNAGDAIMQVYDGTKPMDVVSKADNSPVTAADIAAHTVIMDGL   52 EcCysQ
M-LEQVC-------QLARNAGDAIMQVYDGAKPMEYARKQDDSPVTAADIAAHTVILEGL   52 StCysQ
MALERELLVATQAVRKASLLTKRIQSEVISHKDSTTITKNDNSPVTTGDYAAQTIIINAI   60 ScHAL2
                                          D-----D----------- Consensus1

KEKYPSHSFIGEESVAAGEKSILTD--NPTWIIDPIDGTTNFVHRFPF-VAVSIGFAVNK  115 HuIMP
RKSYPQHTIITEES---GELEGTDQ--DVQWVIDPLDGTTNFIKRLPH-FAVSIAVRIKG  109 SuhB
RTLTPDVPVLSEED--PPGWEV-RQHWQRYWLVDPLDGTKEFIKRNGE-FTVNIALIDHG  108 EcCysQ
RTLTPDIPVLSEED--PPAWEV-RQHWQRYWLVDPLDGTKEFIKRNGE-F            98 StCysQ
KSNFPDDKVVGEES--SSGLEGGRKG--RFWCLDPIDGTKGFL-R-GEQFAVCLALIVDG  169 ScHAL2
-----------EE--------------------DPLDG--N.................Consensus1
                    ^55 amino acid insert into scHAL2 (78-133)
KIEFGVVYSCVEGKMYTARKGKGAFCNG-QKLQVSQQEDITTKSLLVTELGSSRTP----  170 HuIMP
RTEVAVVYDPMRNELFTATRGQGAQLNGYRLLGSTARDLDGGTILATGFP-FKAKQ----  164 SuhB
KPILGVVYAPVMNVMYSAAEGKAWKEECGVRKQIQVRDARPPPLVVIS--RSHADA----  162 EcCysQ
VVQLGCIGCPNLVLSSYGAQDLKGHESFGYIFRAVRGLGAFFYSPSSDAESWTKIHVRHL  229 ScHAL2


-------------------ETVRMVLSNMEKLFCIPVHGIRSVGTAAVNMCLVATGGADA  211 HuIMP
-------------------YATTY-INIVGK-LFNECADFRRTGSAALDLAYVAAGRVDG  203 SuhB
-------------------ELKEY-LQQLGE------HQTTSIGSS-LKFCLVAEGQAHV  195 EcCysQ
DTKDMITLEGVEKGHSSHDEQTA-IKNKLNI------SKSLHLDSQ-AKYCLLALGLAJP  281 ScHAL2
                                       GARYVG.............. Consensus3


YYEMG-IHCWDVAGAGIIVTEAGGVLMDV-TGGPFDLMSRRVIAA---NNRILAERIAK   266 HuIMP
FFEIG-LRPWDFAAGELLVREAGGIVSDF-TGGH-NYMLTGNIVA---GNP----RVVK   253 SuhB
YPRFGPTNIWDTAAGHAVAAAAGAHVHDW-QGKPLDYTPRESFLN---PGF----RVSI   247 EcCysQ
IKLSYQEKIWDHAAGNVIVHEAGGIHTDAMEDVPLDFGNGRTLATKGVIASSG-PRELH   340 ScHAL2
         WD-AAG-AI--EAGG                                      Consensus2
              A  V  A
                 L

EIQVIPLQRDDE.....................................................  278 HuIMP
AM--LANMRDELSDALKR...............................................  269 SuhB
Y................................................................  248 EcCysQ
DLVVSTSCDVIQSRNA.................................................  356 ScHAL2
```

Figure 5.5.. Alignment of protein sequences homologous to CysQ.

HuIMP is human inositol monophosphatase. SuhB is from *E. coli suhB* (both Neuwald *et al.* 1991). EcCysQ is *E. coli cysQ* sequence (Neuwald *et al.* 1992). St CysQ is putative *S. typhimurium cysQ* from the *cpdB* DNA sequence (Liu and Beacham 1990). ScHal2 is from *HAL2*, also called *met22*, from *S. cerevisiae* (Gläser *et al.* 1993). Lines under ScHAL2 indicate regions of homology with CysQ. A 55 amino acid fragment of ScHAL2 (78-133) has been excluded for clarity. Consensus sequences 1, 2 and 3 are from Zhang *et al.* (1993), and represent three motifs present in phospatases of the Inositol monophosphatase, Fructose-1,6-bisphosphatase family. Letters in bold have been shown by X-ray crystallography to be required for metal binding in the active site, although the serine in consensus 3, may be required for dimer formation.
Numbers to the right of sequences are amino acid residue numbers.
Amino acid codes are given in addendix IV.